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Dark-chilling Effects on C_4 Photosynthesis: Comparison between Grasses with different Decarboxylating Mechanisms

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Ana Sofia Contreiras soares

Junho de 2008

Ao Filipe, aos meus pais, à minha irmã e à Lenita

O caminho faz-se caminhando.
(António Machado, poeta espanhol, 1875-1939)

Os dias prósperos não vêm ao acaso; são granjeados, como as searas, com muita fadiga e com muitos intervalos de desalento.
(Camilo Castelo Branco, escritor português, 1825-1890)

A prática é um professor excepcional.
(Plínio, o Jovem, físico, escritor e orador romano, 61-112)

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Abstract

Climate changes are expected to occur with increase in the mean global temperature by 1.8-4.0ºC. However, low temperatures at night may occur, increasing the damages on the following warm day metabolism. Therefore this Thesis aims to study the effects of a short-term dark-chilling on photosynthesis of C_4 plants from different metabolic subtypes, *Paspalum dilatatum* Poiret (NADP-ME), *Cynodon dactylon* (L.) Pers (NAD-ME) and *Zoysia japonica* Steudel (PEPCK). Results showed that the effects of dark-chilling in photosynthesis and leaf parameters on the subsequent warm period depend on the number of chilling nights and on the species studied. In general, a recovery or a tendency to recover after the second consecutive night-chilling was observed and *C. dactylon* showed a less sensitive photosynthesis to night-cold. Furthermore, the damages induced by one night-chilling depend on the light intensity on the subsequent warm day. Although the stress did not alter photosynthesis when measured at low light intensity in any of the species, it has altered the *P. dilatatum* and *Z. japonica* photosynthesis at moderate and high light intensities. One night-chilling also led to an accumulation of carbohydrates and a tendency to an increase in lipid unsaturation degree in all species. At the membrane level, *Z. japonica* seems to be the species better prepared to face chilling. Carboxylating enzymes were changed by stress, but the responses were different among species. A deeper study of *P. dilatatum* photosynthesis revealed a different regulation of photosynthesis and stomatal conductance on each leaf surface, with the adaxial surface presenting a higher decrease of photosynthesis after dark-chilling. Furthermore, differences were also found with light orientation to the leaf, being the photosynthesis on the whole leaf and on the adaxial surface more affected by dark-chilling under adaxial illumination.

Keywords: abaxial surface, adaxial surface, C_4 photosynthesis, dark-chilling, leaf fatty acids.

Sumário

O relatório de 2007 do Painel Intergovernamental em Alterações Climáticas da União Europeia (IPCC) prevê que a temperatura média global no planeta Terra irá subir cerca de 1.8-4.0ºC neste século. No entanto, episódios de baixa temperatura nocturna poderão continuar a ocorrer, prevendo-se que os danos causados pelo frio no metabolismo das plantas irão ser maiores. Temperaturas nocturnas baixas posteriores a e/ou seguidas de um período diurno relativamente quente ou ameno, podem afectar a produtividade das plantas, e em última análise, a economia mundial. Assim, o estudo da resposta das plantas após um período nocturno a baixa temperatura revela-se de extrema importância. Apesar de alguns estudos de frio nocturno terem sido já realizados em plantas com metabolismo fotossintético em C_3, poucos estudos existem em plantas com metabolismo em C_4. O esperado aumento na temperatura média à superfície da Terra poderá levar ao aumento do número de espécies C_4 em regiões não tropicais ou semi-tropicais, assumindo o estudo dos efeitos do frio nocturno no metabolismo de plantas C_4, cada vez mais, uma maior importância.

O objectivo principal desta Tese é estudar os efeitos de um curto período de frio nocturno na fotossíntese e no metabolismo de plantas C_4 no período diurno seguinte. Foram estudadas três plantas C_4, pertencentes aos três subtipos metabólicos, *Paspalum dilatatum* Poiret (NADP-ME), *Cynodon dactylon* (L.) Pers (NAD-ME) and *Zoysia japonica* Steudel (PEPCK). As plantas foram crescidas a uma temperatura de 25ºC, um fotoperíodo de 16/8 h (dia/noite) e a uma intensidade de luz de 250-300 μmol m^{-2} s^{-1}. A baixa luz de crescimento foi escolhida com o intuito de evitar os efeitos concomitantes do frio nocturno e de elevada radiação no período diurno seguinte, uma vez que o objectivo é verificar os efeitos do frio por si só. O stress de frio no período nocturno foi imposto transportando as plantas no fim do período diurno da câmara de crescimento para uma câmara fria (5±3ºC), onde ficaram à escuras até ao início do período diurno seguinte, retornando nessa altura para a câmara de crescimento a 25ºC. Os ensaios fisiológicos e bioquímicos foram realizados entre uma a cinco horas após o regresso das plantas à câmara de crescimento a 25ºC, sendo os resultados consistentes durante este período de tempo.

Nos primeiros ensaios realizados foram determinados parâmetros de folha e trocas-gasosas em plantas sujeitas a uma e a duas noites consecutivas de frio, os quais permitiram escolher o período de frio nocturno a que as plantas iriam ser submetidas nos estudos seguintes. Os resultados mostraram que o efeito do frio nocturno no subsequente metabolismo diurno depende do número de noites a baixa temperatura e da espécie em estudo. Apesar de algumas variações entre as espécies, uma noite de frio levou à alteração de alguns parâmetros estudados, nomeadamente a um aumento da razão peso seco/peso fresco e a uma diminuição da área específica foliar, da taxa de fotossíntese e da condutância estomática. Na segunda noite de frio a maioria dos parâmetros recuperaram ou tenderam a recuperar para valores controlo, indicando que as plantas estão a responder ao stress de frio procurando um novo equilíbrio homeostático. Uma vez que os efeitos foram mais pronunciados após uma noite de frio, foi escolhido este tempo de stress para aprofundar o estudo dos danos causados no metabolismo diurno seguinte.

Os ensaios seguintes revelaram que após uma noite de frio o decréscimo da taxa de fotossíntese era dependente da intensidade de luz no período diurno seguinte. Enquanto que a baixa intensidade luminosa (200 μmol m^{-2} s^{-1}) a taxa de fotossíntese não foi alterada, esta decresceu a luz moderada (530 μmol m^{-2} s^{-1}) e a luz intensa (1300 μmol m^{-2} s^{-1}) em *P. dilatatum* e *Z. japonica* e a luzes muito elevadas (superiores a 1300 μmol m^{-2} s^{-1}) em todas as espécies. Estes resultados indicam que o frio nocturno altera a capacidade das plantas responderem a um aumento da intensidade luminosa, revelando o efeito sinergístico do frio nocturno e da intensidade luminosa. Por este motivo, e uma vez que o objectivo da Tese é verificar os efeitos do frio *per si*, foi escolhido realizar os ensaios bioquímicos à baixa luz de crescimento das plantas (250-300 μmol m^{-2} s^{-1}). Os resultados obtidos indicam que *C. dactylon* é a espécie menos sensível em termos de metabolismo fotossintético a um período curto de frio nocturno. Os resultados sugerem ainda que a taxa de fotossíntese pode ser limitada por efeitos estomáticos em *P. dilatatum* e *Z. japonica* para qualquer das intensidades de luz estudadas. Apesar de a taxa de transporte electrónico decrescer nestas duas espécies a luz moderada e intensa, apenas limita a fotossíntese em *P. dilatatum* a elevada intensidade luminosa. Os parâmetros de fluorescência indicam ainda que uma noite de frio afecta o transporte electrónico em *P. dilatatum* e *Z. japonica* ao nível da separação de cargas no centro de reacção do fotossistema II.

As três espécies mostram efeitos semelhantes nos ácidos gordos em resposta a uma noite de frio, nomeadamente um aumento do conteúdo em ácidos gordos totais e uma tendência para um aumento da insaturação lipídica. O aumento do conteúdo dos ácidos gordos após uma noite de frio poderá justificar o aumento da integridade membranar observada nas três plantas. O maior aumento da integridade membranar observada na espécie *Z. japonica* pode ser ainda justificado por uma diminuição da peroxidação lipídica nesta espécie após a aplicação do stress, o que sugere que o metabolismo antioxidante desta espécie estará mais activo do que nas outras espécies, onde se observa um aumento da peroxidação lipídica em condições de stress. Além da diferença referida em cima, as três espécies apresentam uma composição diferente de ácidos gordos em plantas controlo, sendo *Z. japonica* a espécie que apresenta um maior índice de duplas ligações enquanto que *C. dactylon* é a espécie que apresenta o menor índice. No seu todo, os resultados dos ácidos gordos, da integridade membranar e da peroxidação lipídica sugerem que, contrariamente aos resultados de trocas-gasosas, a espécie *Z. japonica* é a menos sensível a um período de frio nocturno a nível membranar, enquanto que a espécie *C. dactylon* é a mais sensível.

O conteúdo em carbohidratos solúveis e insolúveis aumentou nas três espécies no período diurno em resposta ao stress de frio nocturno. A acumulação de açúcares pode justificar a diminuição da taxa de fotossíntese após uma noite de frio medida com as intensidades de luz moderada e intensa nas espécies *P. dilatatum* e *Z. japonica*. O mesmo não acontece em *C. dactylon*, uma vez que a taxa de fotossíntese não é afectada pelo frio a não ser a intensidades de luz muito elevadas. Por outro lado, a maior quantidade de carbohidratos, principalmente amido, em todas as espécies após a indução do stress pode justificar a diminuição da área específica foliar e o aumento da razão peso seco/peso fresco após o stress.

Apesar de a taxa de fotossíntese medida à baixa luz de crescimento não ter sido alterada nas três espécies em estudo após uma noite de frio, a actividade das enzimas carboxilativas fosfo*enol*piruvato carboxilase (PEPC) e ribulose-1,5-bisfosfato carboxilase/oxigenase (Rubisco) sofreu alteração. A actividade fisiológica da PEPC aumentou em todas as espécies após uma noite de frio, enquanto que a actividade inicial da Rubisco decresceu em *P. dilatatum*, não variou em *C. dactylon* e aumentou em *Z. japonica*. Apesar da menor actividade inicial da Rubisco em *P. dilatatum*, esta foi suficiente para justificar a taxa de fotossíntese. Ao contrário das outras espécies, que

não revelaram alterações na actividade máxima da PEPC e na actividade total da Rubisco, as plantas de *C. dactylon* sujeitas a uma noite de frio revelaram um aumento das referidas actividades, sugerindo um aumento da quantidade das respectivas proteínas.

A caracterização da fotossíntese e da resposta estomática em cada superfície foliar de *P. dilatatum* revelou que esta monocotiledónea apresenta uma regulação dorso-ventral da fotossíntese e da condutância estomática. A diferente orientação da luz para a superfície adaxial ou abaxial alterou a taxa de fotossíntese e a abertura estomática, tanto na folha inteira como em cada superfície separadamente. A menor sensibilidade dos estomas à luz na superfície adaxial, quase fechando completamente quando a folha é iluminada pela superfície abaxial, pode estar relacionada com a quase nula taxa de fotossíntese na superfície adaxial e a menor taxa de fotossíntese na folha inteira com iluminação abaxial da folha.

A diferente regulação da taxa de fotossíntese em cada superfície foliar, com uma taxa de fotossíntese geralmente igual ou superior na superfície abaxial, não está relacionada com diferenças ao nível da estrutura foliar, da distribuição de proteína PEPC e Rubisco e/ou da absorção, reflexão e transmissão de luz em ambas as superfícies. No entanto, poderá estar relacionada com a diferente sensibilidade dos estomas à luz e com a diferente razão entre a área total das células da bainha do feixe e a área total da primeira camada das células do mesófilo que as rodeiam, por cada vaso condutor, nas duas superfícies. Esta razão é maior na superfície abaxial, podendo levar a um mais rápido fluxo de metabolitos entre os dois tipos de células nesta superfície, originando maiores taxas de fotossíntese. Diferenças entre o estado de activação da PEPC nas duas superfícies são também de considerar.

A aplicação de um stress de frio no período nocturno de uma noite levou a um maior decréscimo da taxa de fotossíntese da folha inteira quando a luz foi orientada para a superfície adaxial, o que poderá estar relacionado com uma diminuição nas actividades máximas da PEPC, da Rubisco e da cadeia de transporte de electrões estimadas através da aplicação do modelo fotossintético para as plantas C_4. A taxa de fotossíntese na superfície adaxial parece ter sido mais afectada pelo stress de frio nocturno do que na superfície abaxial, uma observação mais evidente quando a folha é iluminada pela superfície adaxial. Os resultados apresentados nesta Dissertação revelam que as duas superfícies foliares da espécie monocotiledónea C_4 *P. dilatatum* devem

possuir características bioquímicas e/ou metabólicas diferentes, permitindo a evolução de uma nova área de estudo dentro das plantas C_4.

Palavras-chave: ácidos gordos foliares, fotossíntese em plantas C_4, frio nocturno, superfície abaxial, superfície adaxial.

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List of abbreviations

A	Net CO_2 assimilation rate
ABA	Abscisic acid
ADP	Adenosine 5'-diphosphate
Ala	Alanine
Amax	Maximal rate of photosynthesis estimated from photosynthetic curves
AMP	Adenosine 5'-monophosphate
ANOVA	Analysis of variance
Asp	Aspartate
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine 5'-triphosphate synthase
BS	Bundle sheath
BSA	Bovine serum albumin
b6f	Cytochrome b6f
Chl	Chlorophyll
Ci	Intercellular CO_2 concentration
Cm	Mesophyll cells CO_2 concentration
Cs	Bundle sheath cells CO_2 concentration
C_3	Photosynthetic metabolism in which the first stable compound has 3 carbon atoms
C_4	Photosynthetic metabolism in which the first stable compound has 4 carbon atoms
C16:0	Palmitic acid
C17:0	Heptadecanoic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
DBI	Double bound index
DNS	Dinitrosalicylic acid
DTT	Dithiothreitol
DW	Dry weight
EC	Enzyme commission (number)
ETR	Electron transport rate
EDTA	Ethylenediaminetetracetic acid
f	Factor that corrects for spectral quality of light
FBPase	Fructose-1,6-bisphosphatase
Fe	Iron
Fv/Fm	Maximum photochemical efficiency of PSII reaction centres of dark-adapted leaves
FW	Fresh weight
gbs	Bundle-sheath conductance to CO_2
gi	Mesophyll conductance to CO_2

gs	Stomatal conductance to water vapour
h	Hour
HCO_3^-	Bicarbonate ion
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
I	Irradiance used
Ia	Total absorbed irradiance
Jmax	Maximal electron transport rate estimated from photosynthetic curves
Jt	Predicted electron transport rate from photosynthetic curves
K_c	Michaelis-Menten constant of Rubisco for CO_2
kDa	Kilodalton
K_o	Michaelis-Menten constant of Rubisco for O_2
K_p	Michaelis-Menten constant of PEPC
LRWhite	London resin white
M	Mesophyll
MDA	Malondialdehyde
MDH	Malate dehydrogenase
min	Minute
mRNA	Messenger ribonucleic acid
NAD	β-nicotinamide adenine dinucleotide
NADH	β-nicotinamide adenine dinucleotide, reduced
NADP	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced
NAD-ME	NAD-malic enzyme
NADP-ME	NADP-malic enzyme
NAD-MDH	NAD-malate dehydrogenase
NADP-MDH	NADP-malate dehydrogenase
NPQ	Non-photochemical quenching coefficient
O	O_2 partial pressure in the bundle sheath and mesophyll cells
OAA	Oxaloacetate
PBS	Phosphate-buffered saline
PEG	Polyethilenoglycol
PEP	Phospho*enol*pyruvate
PEPC	Phospho*enol*pyruvate carboxylase
PEPCK	Phospho*enol*pyruvate carboxykinase
PG	Phosphatidylglycerol
Pi	Inorganic phosphate
PMSF	Phenylmethylsulphonyl fluoride
PPdK	Pyruvate phosphodikinase
PPFD	Photosynthetic photon flux density
PPi	Pyrophosphate
PSI	Photosysthem I
PSII	Photosysthem II
PVP	Polyvinylpyrrolidone

Q_A^-	Reduced Quinone A
qP	Photochemical quenching coefficient
Q_{10}	Temperature coefficient that represents the factor by which the rate of a reaction increases for every 10ºC rise in temperature
R	Pearson correlation coefficient
RbcS	Small sub-unit of Rubisco
Rd	Mitochondrial respiration estimated from photosynthetic curves
Rm	Mesophyll mitochondrial respiration estimated from photosynthetic curves
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-bisphosphatase carboxylase/oxygenase
RuBP	Ribulose bisphosphate
RWC	Leaf relative water content
s	Second
SBPase	Sedoheptulose-1,7-biphosphatase
SD	Standard deviation
SE	Standard error
SLA	Specific leaf area
SOD	Superoxide dismutase
SPS	Sucrose phosphate synthase
SPSS	Statistical package for social sciences
TBA	Trichloroacetic acid
TBARS	Thiobarbituric acid reacting substances
TCA	Tricarboxylic acid
TP	Triose phosphate
Tukey HSD	Tukey honest significant differences
TW	Turgid weight
VAZ	Xanthophyll cycle; V is violaxanthin, A is antheraxanthin, Z is zeaxanthin
Vcmax	Maximal Rubisco carboxylation rate estimated from photosynthetic curves
Vi	Initial activity (Rubisco)
Vmax	Maximal activity (PEPC)
Vphysiol	Physiological activity (PEPC)
Vpmax	Maximal PEPC carboxylation rate estimated from photosynthetic curves
Vt	Total activity (Rubisco)
v/v	Volume/volume
v/v/v	Volume/volume/volume
w/v	Weight/volume
x	Partitioning factor of electron transport rate
ϕ	Apparent quantum yield estimated from photosynthetic curves
ϕ_{PSII}	Effective quantum yield of PSII
γ^*	Half the reciprocal of Rubisco speficity

θ	Curvature degree estimated from photosynthetic curves

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Chapter 1.

General Introduction and aims of the Thesis.

1. General introduction and aims of the Thesis.

A plant species growing and reproducing successfully over a long period in a particular environment may be regarded as broadly adapted to the physical and chemical characteristics of that environment (Lawlor 2001). However, whenever they grow, the environmental conditions may change tending to restrict their chances of development and survival (Larcher 2003). If the environmental condition exceeds the upper or lower limit for regulation of physiological and/or metabolic plant processes, it constitutes a stress. Thus, stress is not a characteristic of the environment, but of the plant's ability to deal with it (Lawlor 2001), i.e. an environment that is stressful for one plant may not be for another one.

Stresses play a major role in determining plant distribution, growth and productivity (Boyer 1982). The main stresses encountered by plants are associated with extreme temperature, light, water supply and chemical composition of the environment, as nutrients and heavy metals.

1.1. The stress concept

Stress is usually defined as an external factor that exerts a disadvantageous influence on the plant (Taiz and Zeiger 1998). The environmental changes are sensed primarily by leaves (Dickson and Isebrands 1991), although roots may also have a determinant role.

Plants may be stress resistant or stress sensitive. Among the stress resistant species, some are capable to tolerate while other tend to avoid the stress (Mundree *et al*. 2002, Chaves *et al*. 2003). Stress tolerance can be defined as alterations that lead to the achievement of a new thermodynamic equilibrium but no injury occurs or if injury occurs it is repaired. Stress avoidance occurs when the plant exclude the stress by means of physical or metabolic barriers (Levitt 1980).

The stress can persist on the environment for short- or long-periods. Short-term stress provokes injury in sensitive plants, while long-term stress may lead to plant acclimation to the new environment. Acclimation is usually defined as a nonheritable

modification caused by exposure to new environmental conditions (Hale and Orcutt 1987). The acclimation of plants to changing environmental factors or stresses involves both short-term physiological response and long-term physiological, structural and morphological modifications. These changes help to minimize stress in the plant and to maximize the use of internal and external resources (Dickson and Isebrands 1991). The term adaptation has also been used in the literature to indicate acclimation. However, acclimation can be distinguished from adaptation, which usually refers to the genetically determined level of resistance acquired by a process of selection over many generations (Taiz and Zeiger 1998).

1.2. The plant stress responses

The imposition of a stress generally provokes changes in physical, biochemical, physiological or developmental processes. Some responses to stress may start quickly, whereas others may be initiated only after intervals of hours or days (Geiger and Servaites 1991). The intensity of the response of individual plants to a particular stress factor may depend on the species and its sensitivity to the stress, as well as on the plant developmental age and its seasonal and diurnal metabolic activity (Larcher 2003).

Early responses to stress are restricted to the plant current structures and physiological capabilities. Later responses involve new metabolic and structural capabilities conferred by altered gene expression (Geiger and Servaites 1991), allowing the plants to attain a new homeostatic equilibrium. The first responses are likely to be rapid, helping to ensure survival, whereas the later ones enable plant to develop new physiological capabilities and/or a new morphology, which may restore the previous plant functioning even while the stress persists.

1.3. Temperature stress: a major environmental factor

Temperature is one of the major environmental factors that determine the natural distribution of plants, as well as crop vigour and yield (Berry and Björkman 1980). Each process in plant metabolism, including photosynthesis, is geared to an optimal

temperature, above and below which its performance drops. Depending on the level of temperature stress either the photosynthetic function returns to the previous level of activity or it is seriously impaired at both structural and functional levels (Hale and Orcutt 1987, Larcher 1995).

The temperature stress may result from high or low temperatures, the later being divided in chilling and freezing. The high temperature stress, also known by heat stress, accelerates the movement of molecules, weakening their bonds with macromolecules and turning the lipid layers of biomembranes more fluid. In contrast, the low temperature stress, also known as cold stress, turns the biomembranes more rigid and increases the energy required for activating biochemical processes (Larcher 2003). Heat and cold stresses, depending on their intensity and duration, impair the metabolic activity, growth, and viability of plants and thus set limits to the distribution of species.

1.3.1. The low temperature stress: its importance and types of stress

Cold stress is one of the most serious abiotic stresses that plants have to cope with through their entire life cycle. Due to the expected global temperature increase by a further 1.8-4.0°C this century (IPCC 2007), the cold damages caused by late frost events in early springs, sudden lowering in temperature during winter, low night temperatures after a mild day and low snowfalls in winter are expected to increase (Bracale and Coraggio 2003).

Cold stress has a strong limiting effects on (1) the geographic distribution of wild and crop species, hindering the human necessity to grow some crops outside the limits imposed by their natural distribution; (2) the agronomic yield, reducing the plant growth with negative and unforeseeable effects on the biomass; (3) the product quality, decreasing synthesis, accumulation and storage of proteins and polysaccharides (Bracale and Coraggio 2003).

Cold stress occurs when the temperature is lower than the minimum temperature required for plants best performance. Chilling occurs at above-zero temperatures while freezing occurs at negative temperatures. Both chilling and freezing stresses share some injuries due to the direct effect of low temperature on cellular processes such as lower enzymatic activities and fatty acid fluidity. Additionally to the direct effects, freezing

has an important indirect damage due to ice crystal formation (for review see Smallwood and Bowles 2002 and Bracale and Coraggio 2003).

Only the effects of short-term dark-chilling on photosynthesis on the following warm day are within the scope of this Thesis. However, due to the scarcity of studies on the dark-chilling effects, the introduction will also focus the short-term responses of plants to chilling during the day and night periods.

1.3.2. The chilling stress injury symptoms

Among chilling-sensitive plants two types can be distinguished, the totally and the partially sensitive species (Larcher 2003). In the former, every part of the plant is susceptible to damage, while in the later just some organs or processes are damaged by cold (e.g. flower primordial or ripening fruits). Furthermore, there are also remarkable differences in sensitivity between the organs and tissues of the same plant (Larcher 2003). Also, certain stages in the life cycle of a plant (such as seed imbibition, germination and senescence) may be more sensitive to cold than others.

In addition, there is a great complexity in the responses of plants to low non-freezing temperatures, which rises from the genetic background of species and cultivars, severity and duration of the stress, rate of cooling, seasonal and diurnal plant activities, air humidity, water soil availability, wind presence (causing dehydration), light intensity (causing photoinhibition), as well as the plant organ subjected to the stress and the plant developmental stage (Lyons 1973, Nishida and Murata 1996, Bracale and Coraggio 2003, Larcher 2003).

Typically chilling injury occurs in species of tropical and subtropical origin (Lyons 1973, McWilliam 1983, Lambers *et al.* 1998, Larcher 2003), such as maize (*Zea mays* L.), sorghum (*Sorghum* sp.), cotton (*Gossypium* sp.), soybean (*Glycine max* L.), rice (*Oryza* sp.) and tomato (*Lycopersicumon esculentum* Mill.). However, plants from temperate areas may also show some chilling sensitivity, e.g. spring cereals. For many sensitive plants, injury happens when they are exposed to temperatures of about 10-12ºC, although this generalization does not apply to all cases, because it depends on the species origin (e.g. Lyons 1973).

Several sites of chilling injury have been described in the literature (for review see Lyons 1973 and Bracale and Coraggio 2003). At the whole plant level, cold induces both structural (e.g. increase of leaf thickness) and developmental changes (e.g. pollen maturation, loss of plant vigour, reduction of grow rates). Also, a number of physiological responses occur under cold stress: decreases on the photosynthetic and respiratory rate, changes in the enzymes activity and levels of growth regulators, with an increase of abscisic acid (ABA) and a decrease of gibberellin levels. At the ultrastructural and molecular level, cold alters the organization of chloroplasts and mitochondria and affects several molecular structures. The major cellular damage is at membrane level, due to changes in fatty acid fluidity and to the reaction of these molecules with reactive oxygen species (ROS) (i.e. toxic reactive molecules that rapidly loose an electron as singlet oxygen, superoxide, hydrogenperoxyde and the hydroxyl radical).

1.3.2.1. Membrane level

Chilling responses depend on a common primary mechanism involving loss of membrane function. Low temperature induces a phase transition, from a fluid-crystaline to a gel-like state, in the membranes of chilling-sensitive plants (Murata and Yamaya 1984, Cossins 1994, Vigh *et al.* 1998; for review see Lyons 1973, Nishida and Murata 1996 and Murata and Los 1997), with consequent disorders of processes at membrane level. In the gel phase, lipids are closely packed and more highly ordered which prevents normal physiological functions and can turn the membrane more permeable and prone to rupture. For example, the physical properties of the lipids greatly influence the activities of the integral membrane proteins, including H^+-ATPases (adenosine 5'-triphosphate synthase), carriers, and channel-forming proteins that regulate the transport of ions and other solutes, and enzymes from which metabolism depends (for review see Nishida and Murata 1996).

1.3.2.1.1. Membrane fatty acids unsaturation

Many organisms have developed mechanisms to maintain the appropriate fluidity of membrane lipids in response to the ambient temperature, which is essential to plant

survival (Lyons *et al.* 1964). These mechanisms include changes in the proportions of types of lipids and alterations in the lipid/protein ratio (Klein *et al.* 1999). The most widely recognized change in cell membranes at low temperatures is the unsaturation of lipid acyl chains. The variation of the fatty acid composition of membrane phospholipids allows membranes to keep lipids with a constant fluidity at the temperature of growth – a process called "homeoviscous adaptation" (Sinensky 1974). Phospholipids with unsaturated fatty acids have a lower melting point and are more flexibility than phospholipids with saturated acyl chains (Nishida and Murata 1996). Thus, the ability of cells to maintain membrane fluidity through alteration on the degree of unsaturation in their membranes is thought to be one of the prerequisites for survival at low temperature.

A general increase in level of polyunsaturated fatty acids, especially linolenic acid, is observed in most plants during exposure at low temperatures (Wilson and Cawford 1974, Graham and Patterson 1982, Wang *et al.* 2006). For example, a higher percentage of unsaturated fatty acids have been observed after a chilling treatment in *Nicotiana tabacum* (tobacco) (Kodama *et al.* 1995, Orlova *et al.* 2003), warm-season turfgrasses (Samala *et al.* 1998, Cyril *et al.* 2001*a*, Cyril *et al.* 2001*b*, Crill *et al.* 2002, Shang *et al.* 2006), *Saccharomyces cerevisiae* (Rodríguez-Vargas *et al.* 2007), and bacterial species (Nichols *et al.* 2004). The unsaturation level of fatty acids in response to cold is higher in the cold-tolerant species than in the cold-sensitive species (e.g. Cyril *et al.* 2001*b*). A comparative analysis of mitochondria from two maize genotypes selected for their tolerance to cold germination revealed a higher percentage of 18-carbon unsaturated fatty acids and a higher cytochrome *c* oxidase activity in the mitochondrial inner membranes of the cold-tolerant population (De Santis *et al.* 1999). Furthermore, the chilling resistance of higher plants is known to be positively correlated with the levels of *cis*-unsaturated fatty acids in phosphatidylglycerol (PG) from chloroplast membranes (Murata *et al.* 1982, Murata 1983; for review see Nishida and Murata 1996).

Unsaturation may be achieved either by desaturase enzymes which modify existing lipids or through *de novo* synthesis that incorporates unsaturated lipids directly into the membrane (Ohlrogge and Browse 1995). While the former mechanism provides a potentially more rapid response to a sudden change in temperature, the *de novo* synthesis provides an appropriate mechanism for an adaptation to a permanently cold

environment. Fatty acids desaturases are the enzymes that introduce double bounds into fatty acids (Los and Murata 1998).

Unsaturated fatty acids role in chilling tolerance can be demonstrated using *fad* mutants of *Arabidopsis* plants (for review see Ohlrogge and Browse 1995 and Nishida and Murata 1996), which are defective in desaturation of membrane lipids. These mutants present reduced amounts of polyunsaturated fatty acids and are more sensitive to chilling. Genetic manipulation of the level of *cis*-unsaturated fatty acids in PG has successfully altered the chilling resistance of tobacco (Murata *et al.* 1992, Sakamoto *et al*. 2003), *Arabidopsis* (Wolter *et al.* 1992) and rice (Yokoi *et al.* 1998). However, the same levels of *cis*-unsaturated fatty acids in PG in some cultivars of rice and four inbred lines of maize resulted in different degrees of chilling resistance (Kaniuga *et al.* 1998, Maeda *et al.* 1999).

As already referred (see Section 1.3.2.1.), the normal functioning of processes linked to membranes, as for example photosynthetic and respiratory electron transport, depends on the fluidity of the leaf membranes. It has been shown that in transgenic tobacco plants an increase in the unsaturation of fatty acids in PG accelerate the recovery process after photoinhibition (Moon *et al.* 1995), a process that is also known to be induced by low temperatures (Nishida and Murata 1996). It is believed that the unsaturation of membrane lipids facilitates the reassembly of the pre-D1 protein with the photosysthem II (PSII) complex or the processing of the pre-D1 protein that yields the mature D1 protein (Kanervo *et al.* 1997; for review see Nishida and Murata 1996). The unsaturation of fatty acids in PG (*circa* 30% increase) has been also shown to improve the photosynthetic capacity of rice plants at 17ºC and 14ºC, but not at 11ºC and 5ºC (Ariizumi *et al.* 2002). This result indicates that the positive effects of lipid unsaturation on the photosynthetic process may depend on the severity of chilling stress.

1.3.2.1.2. Membrane permeability and lipid peroxidation

Chilling induces changes in the physical state of the membranes that are thought to be responsible for the increased leakage of cell electrolytes from the tissue of chilling-sensitive plants (Lyons 1973). Enhanced levels of electrolyte leakage have occured in chilled roots (Liebermann *et al.* 1958, Markhart *et al.* 1979), leaves (Creencia and Bramlage 1971, Wright 1974, Patterson *et al.* 1976, Paull 1981) and fruit tissues

(Tatsumi and Murata 1978). Patterson *et al.* (1976) found two phases of ion leakage at 0ºC less-sensitive *Passiflora* species, a relatively slow initial phase, followed by a rapid loss of most of the electrolyte. In the most chilling-sensitive *Passiflora* species, the two phases could not be separated. Paull (1981) described increased leakage as a rapid process that may start 1 h after chilling exposure in chilling sensitive plants. However, this process is rapidly reversible when plants returned to non-chilled temperatures, being necessary no more than 24 h in common bean (*Phaseolus vulgaris* L.) leaves (Wright 1974) or 36 h in maize seedlings (Creencia and Bramlage 1971). Chilling treatment of transgenic tobacco plants transformed with a desaturase gene showed a higher unsaturation of fatty acids and a decreased electrolyte leakage from membranes (Orlava *et al.* 2003), indicating that lipid unsaturation level has an important effect on membrane permeability.

Chilling also induces an oxidative damage to plant leaves (e.g. Prasad *et al.* 1994*a*). The production of ROS greatly affect membrane lipids, since they react with fatty acids, especially with those presenting a higher unsaturation degree as linolenic acid (Halliwell 1984), increasing lipid peroxidation. This lipid peroxidation decreases the membrane integrity and occurs in several chilling-sensitive species after a chilling treatment (e.g. Kaniuga and Michalski 1978, Wise and Naylor 1987, Zhou *et al.* 2004*a*, Munro *et al.* 2004). An efficient antioxidant metabolism is important to scavenge ROS production, decreasing the chilling induced oxidative effects on membrane lipids. Chilling-resistant species show a higher activity of the anti-oxidative metabolism than chilling-sensitive species, as found in maize plants with different chilling sensitivities (e.g. Prasad 1996, Hodges *et al.* 1997*a*).

1.3.2.2. Ultrastructural level

The general ultrastructural symptoms of cold include swelling and disorganization of both chloroplasts and mitochondria, reduced size and number of starch granules in the chloroplast, dilation of thylakoids and unstacking of grana, formation of small vesicles of chloroplast peripheral reticulum and lipid droplet accumulation (Jagels 1970, Taylor and Craig 1971, Kimball and Salisbury 1973, Wise *et al.* 1983, Wise and Naylor 1987, Ishikawa 1996, Yun *et al.* 1996, Garstka *et al.* 2007). Effects of chilling

temperatures on the nucleus are not as widely reported, either because visible changes rarely occur or they are not so pronounced (for review see Kratsch and Wise 2000).

Given the central role of photosynthesis to plant physiology and its sensitivity to chilling stress (see Section 1.3.2.3.2.), it is not surprising that the chloroplast is commonly the earliest visible site of ultrastructural chilling injury in the plant cell (Kimball and Salisbury 1973). Curiously, chloroplasts in maize leaf mesophyll cells are more susceptible to chilling-induced injury than the chloroplasts in the adjacent bundle sheath cells (Slack *et al.* 1974).

Several factors, such as light intensity, relative humidity and inherent sensitivity of plants to low temperature have been recognise to interact with chilling either enhancing the effect or acting as protection against ultrastructural injury (for review see Kratsch and Wise 2000). High light intensities during chilling greatly exacerbate the ultrastructural injury due to chilling stress alone (e.g. Wise *et al.* 1983). High relative humidity (100%) protects the chloroplasts in both chilling-sensitive and chilling-tolerant species, and the protective effect was enhanced by treatment in the dark (e.g. Wise *et al.* 1983). In general, the more sensitive to chilling the plant is, the sooner and more extensive are the ultrastructural changes (Kimball and Salisbury 1973, Wise *et al.* 1983, Wise and Naylor 1987).

1.3.2.3. Physiological and biochemical level

1.3.2.3.1. Chilling induced water stress

Chilling affects the transport of ions and may reduce water uptake by roots (e.g. Melkonian *et al.* 2004), which may in turn decrease leaf water content. Root growth and function may also be negatively affected by chilling in what concerns ion leakage and root conductivity (Rab and Saltveit 1999 and references therein, Aroca *et al.* 2005). Chilling induced water stress has been shown in several plants (e.g. Hällgren and Öquist 1990, Báló *et al.* 1991, Boese *et al.* 1997, Flexas *et al.* 1999). This process is thought to be caused by a combination of two factors, an increase of water viscosity due to increased hydrogen bonding that reduces water flow through xylem vessels and/or to an increase in the resistance of the roots due to reduced permeability of root cells to water at low temperature (Kramer 1983, Hällgren and Öquist 1990).

In some plants one factor seems to be preponderant in relation to the other. For example, chilling-associated water stress induction in grapevine was present even when roots were not chilled, indicating that the response in grapevine may be dominated by an hydraulic resistance of the stem xylem system (e.g. Schultz and Matthews 1988, Lovisolo and Schubert 1998). On the contrary, water deficit in soybean dark-chilled plants just occur if roots were chilled (van Heerden *et al.* 2003*a*), indicating a preponderant effect of decreased water conductivity by roots. In accordance, mango (*Mangifera indica* L., Allen *et al.* 2000) and coffee (*Coffea arabica* L., Bauer *et al.* 1985) plants dark-chilled at the shoot level also show no effect of low temperature on leaf water content.

1.3.2.3.2. Photosynthetic activity and oxidative damage

Photosynthesis is the physiological process injured by cold that has received more attention in the literature. Photosynthesis shows an optimum temperature and decrease at higher and lower temperatures (for review see Sage and Kubien 2007). The range of temperature at which there is no apparent injury in the rate of photosynthesis varies with species and growth conditions (for review see Sage and Kubien 2007 and references therein), and is loosely defined as the range of temperature at which the photosynthetic rate is fully reversible after short-term periods at non-optimal temperature.

Both the contribution of stomatal and non-stomatal effects to the decrease of photosynthesis under cold temperatures have been observed in chilling sensitive species (for a review see Allen and Ort 2001). Stomatal closure is not surprising since chilling may induce water stress, as referred above (see Section 1.3.2.3.1.).

Cold temperatures also affect negatively the activity of photosynthetic enzymes and the thylakoid electron transport (for a review see Allen and Ort 2001). Inhibition of enzymes of carbon metabolism have been observed in several species subjected to a chilling stress (e.g. Taylor *et al.* 1974, Sassenrath and Ort 1990, Kingston-Smith *et al.* 1997, Du *et al.* 1999*a*). It has been shown that in tomato the inhibition of sedoheptulose-1,7-bisphosphate (SBPase, EC 3.1.3.37) and fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11) closely mirror the chilling-induced inhibition of photosynthesis (Sassenrath and Ort 1990, Sassenrath *et al.* 1990). Other enzymes, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39), phospho*enol*pyruvate

carboxylase (PEPC, EC 4.1.1.31), pyruvate phosphodikinase (PPdK, EC 2.7.9.1), NADP-malate dehydrogenase (NADP-MDH, EC 1.1.1.82) and NADP-malic enzyme (NADP-ME, EC 1.1.1.40) also show reduced activity in cold exposed plants (Taylor *et al.* 1974, Sugiyama *et al.* 1979, Potvin *et al.* 1986, Brunell 1990, Hurry *et al.* 1995, Kingston-Smith *et al.* 1997, Du *et al.* 1999*a*, Du *et al.* 1999*b*; for a review see Guy 1990).

Recently Sage and Kubien (2007) have reviewed the limitations of the C_3 and C_4 photosynthetic cycles to variations in temperature from the CO_2-response curves of photosynthesis. The rate of net CO_2 assimilation in the leaves of C_3 plants is controlled at low CO_2 concentration by the Rubisco capacity and at high CO_2 values by the capacity to regenerate ribulose bisphosphate (RuBP) and the capacity to regenerate inorganic phosphate (Pi) (Harley and Sharkey 1991, von Caemmerer 2000). Sage and Kubien (2007) suggest that at atmospheric CO_2 concentrations the Pi regeneration capacity in the chloroplast is the main limiting factor at low temperature and electron transport limit photosynthesis at high temperature, while Rubisco capacity limits photosynthesis at intermediate temperatures. In the C_4 plants, photosynthesis is dependent on the capacity of PEPC to fix bicarbonate at low CO_2 concentrations (von Caemmerer 2000). At high CO_2 concentrations photosynthesis may be limited by Rubisco capacity, RuBP regeneration capacity, phospho*enol*pyruvate (PEP) regeneration controlled by PPdK capacity, and Pi regeneration capacity. Although the activity of PEPC decreases at low temperature (e.g. Pittermann and Sage 2000, Kubien and Sage 2004), at atmospheric CO_2 conditions the most limiting factor for C_4 photosynthesis under chilling conditions is the Rubisco capacity (for review see Sage and Kuben 2007).

Chilling often leads to photoinhibition and to photooxidation (leaf bleaching) (Nishida and Murata 1996, Lambers *et al.* 1998) because the biophysical reactions of photosynthesis (photon capture and transfer of excitation energy) are less affected by temperature than the biochemical steps, including electron transport and activity of the Calvin cycle. Due to the excess of energy in the photosynthetic electron transport, chilling often increases the production of ROS (e.g. O'Kane *et al.* 1996, Kingston-Smith *et al.* 1999, Pastori *et al.* 2000, Li *et al.* 2003), which may be produced at the PSI level (for review see Lawlor 2001 and Ivanov and Khorobrykh 2003) and at the PSII

level under strong illumination (e.g. Cleland and Grace 1999, Ivanov and Khorobrykh 2003, Song *et al.* 2006).

The oxidative stress induced by chilling has a negative impact on proteins and proteases. Proteins are damaged by oxidation during exposure to stress and proteases are inhibited by oxidants, leading to an accumulation of damaged proteins with impaired functional activities under chilling conditions (Stadtman and Oliver 1991, O'Kane *et al.* 1996). Prasad (1996) found an accumulation of oxidized proteins in maize seedling exposed to 4ºC. *In vitro* experiments with purified proteins or isolated chloroplasts have shown that chloroplast proteins such as Rubisco large sub-unit (Ishida *et al.* 1997), D1 protein (Miyao 1994), CuZn-superoxide dismutase (SOD) (Casano *et al.* 1994) and chloroplast glutamine synthetase (Ishida *et al.* 2002) are directly fragmented by ROS. Nakano *et al.* (2006) showed that the direct fragmentation of the Rubisco large sub-unit by ROS also occurs in intact leaves under chilling in the light and was completely inhibited by ROS scavengers.

Cold-acclimated plants may prevent the formation of ROS, avoiding a photoinhibition process, by dissipation of excess energy through the de-epoxidation of violaxanthin to zeaxanthin in the xantophyll cycle (Bilger and Björkman 1991, Chaumont *et al.* 1995, Haldimann *et al.* 1995, Haldimann 1996, Verhoeven *et al.* 1996). In accordance, the overexpression of the zeaxanthin epoxidase gene (involved in the epoxidation of zeaxanthin to violaxanthin) has been shown to enhance the sensitivity of tomato plants to photoinhibition induced by high light and chilling stress (Wang *et al.* 2008), due to the impairment of the function of the xanthophyll cycle.

Plants may also avoid the damage induced by ROS through an increase in the antioxidant levels and antioxidant enzymes (for review see Foyer *et al.* 2002 and Suzuki and Mittler 2006). Chilling resistance it thought to require an effective up-regulation of the antioxidant system (Foyer and Harbinson 1994, Fryer *et al.* 1998). It has been shown that the ability of maize seedlings to survive depends on their capacity during acclimation to increase the synthesis and the activity of antioxidant enzymes, such as SOD, catalase, and peroxidases (Prasad *et al.* 1994*a*, Prasad *et al.* 1994*b*, Prasad *et al.* 1995, Zhang *et al.* 1995, Prasad 1996, Hodges *et al.* 1997*a*, Hodges *et al.* 1997*b*, Prasad 1997, Aroca *et al.* 2001). Furthermore, glutathione one of the major redox buffers (Noctor and Foyer 1998) has been strongly implicated in chilling tolerance, particularly

in maize plants (Kocsy *et al.* 1996, Kocsy *et al.* 2000*a*, Kocsy *et al.* 2000*b*, Kocsy *et al.* 2001*a*, Kocsy *et al.* 2001*b*, Gómez *et al.* 2004).

1.3.2.3.3. Respiratory activity

As photosynthesis, respiration has also an optimum temperature at which its rate is maximal and has been recognised as a temperature sensitive process. Changes in the fluidity of the membrane that occur under chilling (see Section 1.3.2.1.1.) may alter the normal functioning of the respiratory pathway. Furthermore, at low temperature the respiratory flux is probably limited by the maximal activity of the enzymes involved in the respiratory pathway (i.e. glycolysis, the tricarboxylic acid (TCA) cycle and mitochondrial electron transport) (Atkin and Tjoelker 2003).

Chilling treatment have been shown to lower the expression and activity of cytochrome *c* oxidase in the mitochondrial inner membrane of a chilling sensitive genotype of maize (Stewart *et al.* 1990*a*, Stewart *et al.* 1990*b*, Prasad *et al.* 1994*b*, De Santis *et al.* 1999). However, the observed decline in the activity of this enzyme was partially counterbalanced by an increase in the rate of the alternative oxidase.

1.3.2.4. Other symptoms of chilling injury

Chilling injury is known to cause leaf discolouration due to impairment or lost of chlorophyll or lesions on leaves as observed in the C_4 plant maize (Haldimann *et al.* 1995). Furthermore, in chilling-sensitive rice plants (*Oryza sativa* L.) the low temperature induced chlorosis is associated with chilling-induced suppression of gene expression (Yoshida *et al.* 1996).

A depression of carbohydrate translocation, inhibition of protein synthesis and increased degradation of existing proteins has also been shown to occur under chilling conditions (Potvin *et al.* 1984, Bredenkamp and Baker 1994, Chaumont *et al.* 1995). The accumulation of acethaldehyde, ethanol and significant changes in the content of aminoacids are also among the most common metabolic changes reported as symptoms of chilling injury in sensitive species (Slack *et al.* 1974, Rosinger *et al.* 1984, Du *et al.* 1999*a*).

1.3.3. Dark-chilling stress: its importance and symptoms

The study of the effects of chilling in the dark on the subsequent warm day photosynthesis is pertinent once plants under natural and agricultural habitats experience the lowest temperatures at night. Furthermore, it allows the study of chilling effects by itself without the adding effect of light.

When chilling is imposed in the light, the effects of light intensity in photosynthesis are typically greater and may mask those induced by chilling alone (Allen and Ort 2001). For that reason, there are differences in the degree of the inhibition of photosynthesis and on the main effects of chilling between plants chilled in the light and dark periods or only in the dark period (Allen and Ort 2001, Fig. 1.1.). While the effects of chilling only in the dark involve mainly decreases within the processes of photophosphorylation in the thylakoid membrane, the Calvin-Benson cycle in the stroma and the CO_2 supply to the chloroplast through the stomata, chilling during the 24 h also inhibits greatly the carbohydrate metabolism in the cytosol (Allen and Ort 2001). Furthermore, light-chilling can be regarded as a type of photoinhibition in which oxidative stress plays an important role, whereas the effects of dark-chilling are mainly induced by chilling alone (Garstka *et al.* 2007). In spite of what was described above, the mechanisms involved in the dark-chilling are not clearly defined, especially in C_4 plants.

1.3.3.1. Photosynthetic metabolism

Although the few studies on the effects of dark-chilling on plant physiology and metabolism, it has been shown that short-term chilling in the dark decreased the photosynthetic rate on the subsequent warm day in a manner that is dependent on the species and on the subsequent day light intensity.

Photosynthetic rate has decreased after one to several nights of chilling in some chilling-sensitive species such as tomato (Martin *et al.* 1981), coffee (Bauer *et al.* 1985), mango (Allen *et al.* 2000), cucumber (*Cucumis sativus* L., Jun *et al.* 2001, Zhou *et al.* 2004*b*), grapevine (*Vitis vinifera* L., Flexas *et al.* 1999, Bertamini *et al.* 2005, Bertamini *et al.* 2006) soybean (e.g. van Heerden *et al.* 2003*b*, Strauss *et al.* 2007), tree species (Feng and Cao 2005) and in several C_4 species (Ludlow and Wilson 1971, Pasternak

and Wilson 1972, Ivory and Whiteman 1978, Long *et al.* 1983, Pittermann and Sage 2001). van Heerden *et al.* (2003*b*) have shown that a more chilling sensitive soybean genotype presented a decrease in photosynthetic rate after the first chilling night, whereas the less sensitive genotype just presented a decrease in the CO_2 assimilation rate after the second consecutive chilling night. Furthermore, the negative effect of dark-chilling on photosynthesis was higher when plants were transferred to high light intensities (Feng and Cao 2005). Different responses to dark-chilling have been observed if roots were also subjected to this stress (van Heerden *et al.* 2003*a*).

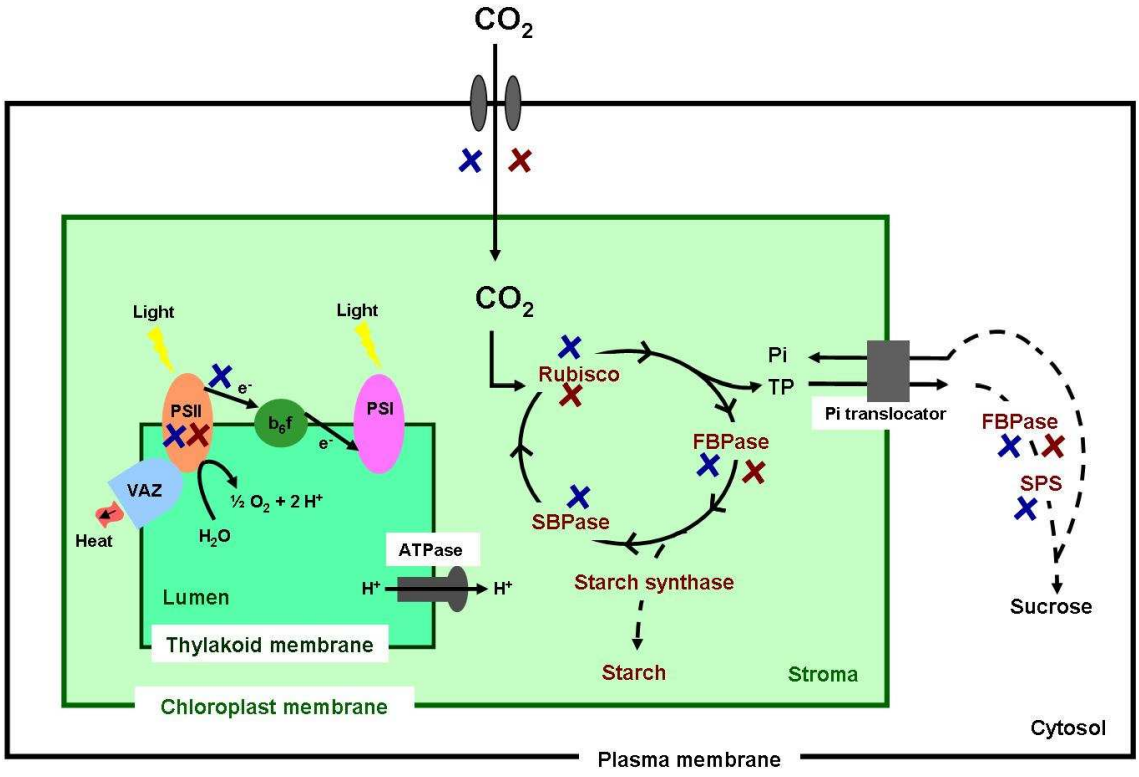

Figure 1.1. Main effects of a short-term chilling stress imposed in the light or only in the dark on the C_3 photosynthetic metabolism. Blue crosses represent the known main impacts of a light-chilling, while red crosses represent the known main impacts of a dark-chilling (Adapted from Allen and Ort 2001).
Abbreviations: ATPase, adenosine 5'-triphosphate synthase; b6f, cytochrome b6f complex; FBPase, fructose-1,6-bisphosphatase; Pi, inorganic phosphate; PSI, photosystem I; PSII, photosystem II; Rubisco, ribulose-1,5-bisphosphatase carboxylase/oxygenase; SBPase, sedoheptulose-1,7-bisphosphatase; SPS, sucrose phosphate synthase; TP, triose phosphate; VAZ, xanthophyll cycle where V is violaxanthin, A is antheraxanthin and Z is zeaxanthin.

Stomatal (e.g. Pasternak and Wilson 1972, Martin *et al.* 1981, Bauer *et al.*, 1985, van Heerden *et al.* 2003*b*, Feng and Cao 2005) and non-stomatal (e.g. Pittermann and

Sage 2001, van Heerden *et al.* 2003*b*, Strauss *et al.* 2007) responses limit photosynthesis after the dark-chilling stress. Among the non-stomatal limitations, both enzymatic and electron transport damages have been reported. Dark-chilling alters the circadian regulation of Rubisco activase mRNA (messenger ribonucleic acid) transcription (Martino-Catt and Ort 1992) and the circadian rhythms in activity of SPS and nitrate reductase (Jones *et al.* 1998) in C_3 plants. Experiments with soybean and cucumber plants grown for several days in a night-chilling/ light-warm cycle showed a decrease in the activity of FBPase and NADP-MDH (Jun *et al.* 2001, van Heerden *et al.* 2003*b*). Results of Rubisco activity are not so consensual. While a decrease in cucumber (Zhou *et al.* 2004*a*), soybean genotype Java 29 (van Heerden *et al.* 2003*a*) and in the C_4 NAD-malic enzyme (NAD-ME) species *Muhlenbergia montana* (Nutt.) (Pittermann and Sage 2001) was observed, Rubisco activity was not affected in the soybean genotypes Maple Arrow and Freskeby V (van Heerden *et al.* 2003*b*).

The PSII reaction centre seems to be a main target of dark-chilling (Kaniuga *et al.* 1978*a*, Martin and Ort 1982, van Heerden *et al.* 2003*a*, van Heerden *et al.* 2003*b*, van Heerden *et al.* 2004, Bertamini *et al.* 2005, Bertamini *et al.* 2006, Strauss *et al.* 2007). Strauss *et al.* (2007) found an accumulation of the reduced Quinone A (Q^-_A) pool and an uncoupling of the oxygen-evolving complex in soybean plants dark-chilled for three consecutive nights and subsequent re-warmed at high light intensity. Bertamini *et al.* (2006) observed a decrease in the content of D1 protein in grapevine plants after one night chilling and a subsequent high light intensity. Furthermore, the decrease in PSII activity in leaves of cold-sensitive plants chilled in the dark for three to five days was associated with the release of manganese from the oxygen-evolving complex of PSII (Kaniuga *et al.* 1978*b*, Higuchi *et al.* 2003) and with the destabilization of PSII extrinsic proteins (Shen *et al.* 1990, Garstka and Kaniuga 1991).

Dark-chilling also inhibited the leaf translocation of photoassimilates at night (Potvin *et al.* 1985). However, in soybean plants dark-chilled for one to three consecutive days starch and sucrose did not accumulate (van Heerden *et al.* 2003*b*).

1.3.3.2. Membrane fatty acids and lipid peroxidation

Studies on membrane fatty acids after dark-chilling are scarce. However, an accumulation of free fatty acids in detached leaves of chilling-sensitive species related

to a high activity of endogenous galactolipase (Gemel and Kaiuga 1987, Kaniuga 1997) has been shown. Furthermore, lipid peroxidation products also increase in response to dark-chilling (Kaniuga and Michalski 1978, Feng and Cao 2005), which is in accordance with the increase in the ROS content and antioxidant enzymes activity in dark-chilled plants (van Heerden and Krüger 2002, Feng and Cao 2005).

1.3.3.3. Chloroplast ultrastructure

Similarly to chilling responses during both light and dark periods (see Section 1.3.2.2.), the chloroplast ultrastructure was affected by dark-chilling in sensitive species of bean and tomato, but not in the cold-tolerant spinach species (*Spinacia oleracea* L., Gemel *et al.* 1986). It is believed that the disturbance of thylakoid ultrastructure induced by dark-chilling might be a consequence of rearrangements of chlorophyll-protein in the light harvesting complexes of PSI and PSII (Garstka *et al.* 2005, Garstka *et al.* 2007).

The damage effects of dark-chilling on chloroplast ultrastructure and metabolism may be reversible within few hours of exposition to warm temperatures at low light intensities (Garstka and Kaniuga 1991, Garstka *et al.* 2007), but the recovery of PSII activity is slower (Garstka *et al.* 2007). However, a long-term dark-chilling at moderate and high irradiance levels during the first hours of the warm light period turn the leaves of chilling-sensitive plants susceptible to photoinhibition due to the non-recovery of PSII activity (Garstka *et al.* 2005).

1.4. C_4 photosynthetic metabolism and chilling sensitivity

The C_4 photosynthetic metabolism (abbreviated from C_4 dicarboxylic acid pathway of photosynthesis) is present in the so-called C_4 species and involves remarkable modifications in leaf anatomy and photosynthetic metabolism, allowing an increase in the CO_2 concentration at Rubisco level in the Calvin-Benson cycle (for review see Furbank and Taylor 1995 and Sage 1999), minimizing the oxygenase activity and consequently photorespiration (Chollet and Ogren 1975). The discovery of the C_4 photosynthetic pathway occurred in the mid-60s in the tropical grasses sugarcane (*Saccharum* sp.) and maize (Kortschak *et al.* 1965, Hatch and Slack 1966), and since

then further discoveries have been found, including the existence of different metabolic pathways in other C_4 plants and the regulation of this photosynthetic process (for review see Hatch 1999).

C_4 photosynthesis may have appeared in response to the prehistoric advent of low atmospheric CO_2 conditions in the Paleocene ($7x10^7$ years ago) and the Miocene ($2x10^7$ years ago), that allowed for significant Rubisco oxygenase activity and photorespiration (Ehleringer *et al.* 1991). At current CO_2 levels, photorespiration can reduce photosynthesis by more than 40% at warmer temperatures in C_3 species (Sharkey 1988, Ehleringer *et al.* 1991). C_4 photosynthesis seems to be restricted to the Angiosperm class, evolved independently at least 31 times and it is now represented by 8,000 to 10,000 species classified in 18 diverse taxonomic families (Sage 2001). The variations on the basic C_4 scheme reflect differences in evolutionary origin of the various C_4 species (Dengler and Nelson 1999, Kellogg 1999).

C_4 plants have important agronomic implications, since they contribute to 30% of the total terrestrial primary productivity (Gillon and Yakir 2001). In addition, the food supply in many tropical regions is largely based on C_4 plants, including gramineae that supply the grains of many tropical diets and pasture and forage for livestock (Brown 1999, Zhang *et al.* 2006*b*). C_4 turf grasses are also extensively used in several athletic fields, playgrounds and public grounds, sustaining for example the multibillion dollar golf industry (Brown 1999, Zhang *et al.* 2006*b*). C_4 plants may also play an important role in the biofuel production (Lawrence and Walbot 2007).

1.4.1. Two different cell types – mesophyll and bundle sheath cells

Most C_4 plants have evolved in their leaves two different types of cells with unique characteristics, the mesophyll (M) and the bundle sheath (BS) cells. Generally these cells are arranged in the so-called Kranz-anatomy, characterized by the M cells surrounding BS cells, which in turn surround the vascular tissues of the leaf (for review see Dengler and Nelson 1999). However, recent works have shown that the Kranz-anatomy is not essential for the C_4 photosynthetic metabolism, being observed in an unicellular diatom (Reinfelder *et al.* 2000) and in three succulent species of the Chenopodiaceae family, *Bienertia cycloptera* Bunge ex Boiss, *Bienertia sinuspersici* Akhani and *Sueda aralocasoica* (Bunge) Freitag and Schütze (formerly classified as

Borszczowia) which does not show Kranz-anatomy (Voznesenskaya *et al.* 2002, Voznesenskaya *et al.* 2003, Akhani *et al.* 2005).

The M and the BS cells work co-ordinately, but present distinct morphological and biochemical functions since the enzymes of the C_4 pathway are located separately (See Section 1.4.2.). The chloroplasts of the M cells are similar to those of C_3 plants, except that they do not present Rubisco and other enzymes that constitute the Calvin-Benson cycle. This cycle is present in the BS cells chloroplasts, which are functionally similar to those of C_3 plants (Kanai and Edwards 1999). The morphological and biochemical differences of the two cell types are clearly seen in the three metabolic sub-types (See Section 1.4.2.).

Although there are two distinct cells types that account for the C_4 photosynthetic metabolism, it was always though that the M and BS cells from the adaxial and the abaxial leaf surface had the same characteristics. However, recent work developed by Driscoll *et al.* (2006) show that photosynthesis in maize, a monocotyledonous C_4 species, is differently regulated on the adaxial and abaxial surfaces as in the dicotyledonous C_3 plants. While in the latter plants the dorso-ventral regulation of photosynthesis is related to the presence of two anatomical and biochemical distinct mesophyll cells on the adaxial and abaxial surface (e.g. Terashima and Inoue 1985*a*, Terashima and Inoue 1985*b*, Evans and Vogelmann 2003), in the C_4 monocotyledonous the different regulation of photosynthesis is surprising due to the symmetrical leaf structure.

1.4.2. Photosynthetic C_4 metabolic subtypes

The basic C_4 photosynthetic pathway consists of four stages: (1) primary fixation of CO_2 by the carboxylation of PEP in the M cells to form a C_4 acid; (2) transport of the C_4 acids to the BS cells; (3) decarboxylation of the C_4 acids within the BS cells; and (4) transport of the C_3 acid, formed by the decarboxylation step, back to the M cell and regeneration of the CO_2 acceptor PEP (for review see Kanai and Edwards 1999).

The primary carboxylation reaction, catalyzed by PEPC, is common to all variants and occurs in the cytosol of the M cells (for revision see Kanai and Edwards 1999). PEPC catalyzes the irreversible β-carboxylation of PEP in the presence of bicarbonate ion (HCO_3^-) and divalent metallic ions, namely Mg^{2+}, forming oxaloacetate (OAA) and

Pi (Lepiniec *et al.* 1994; for revision see Chollet *et al.* 1996). The OAA formed is rapidly metabolized into dicarboxilic acids (malate and/or aspartate), which are transferred to the BS cells and decarboxylated to CO_2 and pyruvate or PEP, depending on the metabolic sub-type. The CO_2 is refixed by Rubisco and assimilated in the Calvin-Benson cycle, while pyruvate, alanine or PEP, depending on the metabolic sub-type, are transported to the M cells. Once in the M cells, pyruvate regenerates PEP in a reaction catalyzed by the chloroplastidial enzyme PPdK (for revision see Kanai and Edwards 1999 and Arrabaça 2008).

There are three major subtypes of the basic C_4 photosynthetic pathway (Fig. 1.2., 1.3. and 1.4.), named accordingly to the enzyme used to catalyze the decarboxylating reaction in the BS cells: (1) NADP-dependent malic enzyme (NADP-ME) in the chloroplasts; (2) NAD-dependent malic enzyme (NAD-ME, EC 1.1.1.39) in the mitochondria; and (3) phospho*enol*pyruvate carboxykinase (PEPCK, EC 4.1.1.32) in the cytosol (for review see Kanai and Edwards 1999). Whereas most C_4 species primarily use only one of the decarboxylating enzymes, a number of species employs a second enzyme in a supporting role (Sage 1999, Wingler *et al.* 1999). For example, PEPCK species usually employ NAD-ME in the BS cells to generate NADH inside the mitochondria. The three metabolic sub-types also present morphological differences in the ultrastructure and arrangement of BS chloroplasts (Gutierrez *et al.* 1974, Hatch *et al.* 1975; for review see Kanai and Edwards 1999).

In the NADP-ME metabolic sub-type, the BS chloroplasts are usually arranged in a centrifugal position relative to the vascular bundle, and have thylakoid membranes with reduced grana stacking (for revision see Kanai and Edwards 1999). Recent work have shown that the polypeptides that constitutes the PSII are present in the BS cells agranal chloroplasts of maize, a NADP-ME species, but in a much lower number than in the M cells (Drozak and Romanowska 2006, Romanowska and Drozak 2006, Romanowska *et al.* 2006). In addition, the activity of PSII is very low in the BS cells (Romanowska *et al.* 2006), which is in accordance with earlier works (Ku *et al.* 1974, Schuster *et al.* 1985, Edwards *et al.* 2001). The PSII presence in the agranal BS chloroplasts may have a role in balancing the redox state of cyclic electron flow around PSI for ATP synthesis (Romanowska *et al.* 2006). The OAA formed in this metabolic sub-type is reduced to malate by NADP-MDH in the M chloroplasts (Fig. 1.2.). The malate is then transported to the BS cells, possibly through plasmodesmata, where is decarboxilated by NADP-

ME in the chloroplast forming CO_2 and pyruvate, the later being transported to M cells (for revision see Kanai and Edwards 1999 and Arrabaça 2008).

NADP-ME subtype

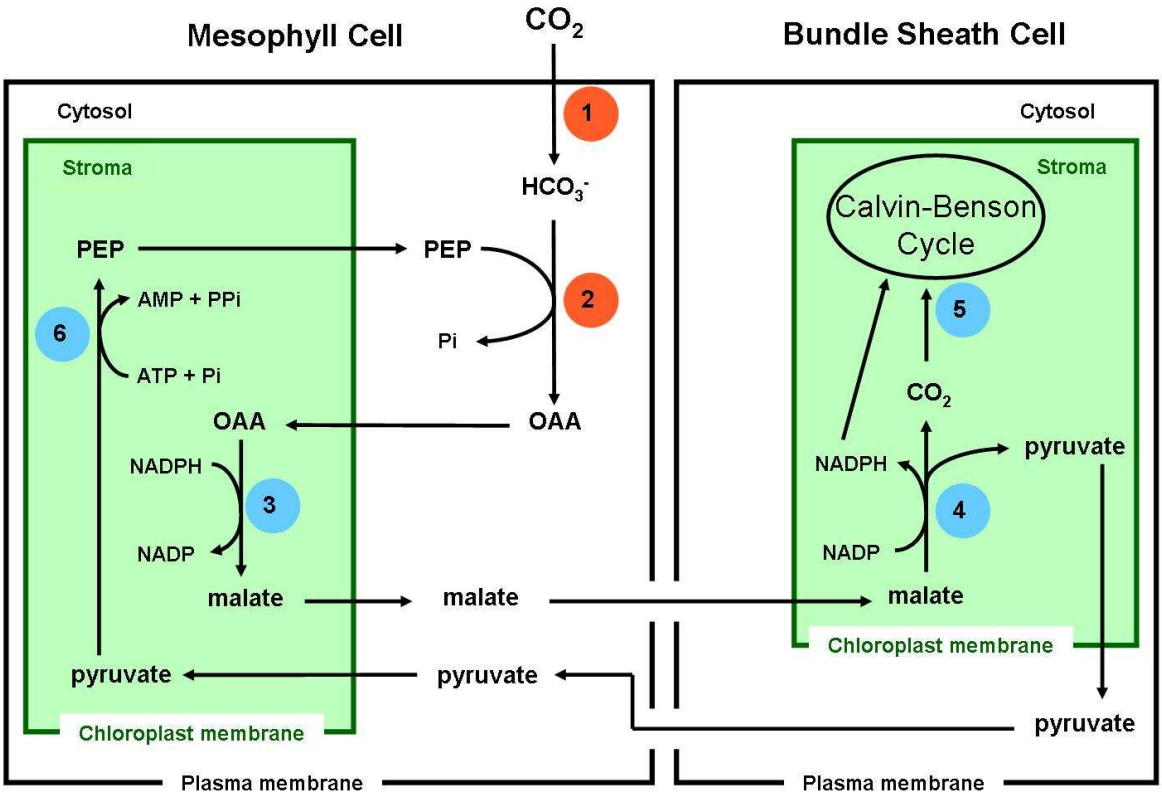

Figure 1.2. Simplified scheme of the key reactions and their location in the mesophyll and bundle sheath cells of a NADP-ME C_4 plant (Adapted from Kanai and Edwards 1999).
Compound abbreviations: AMP, adenosine 5'-monophosphate; ATP, adenosine 5'-triphosphate; HCO_3^-, bicarbonate ion; NADP, β-nicotinamide adenine dinucleotide phosphate; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pi, inorganic phosphate; PPi, pyrophosphate.
Enzyme abbreviations: 1, carbonic anhydrase; 2, PEP carboxylase; 3, NADP-malate dehydrogenase; 4, NADP-malic enzyme; 5, ribulose-1,5-bisphosphate carboxylase/oxygenase; 6, pyruvate phosphodikinase. Enzyme numbers inside red circles correspond to reactions occurring in the cytosol, while inside blue circles correspond to reactions occurring in the chloroplast.

In the NAD-ME metabolic sub-type, the BS chloroplasts have thylakoid membranes with developed grana stacking. Both chloroplast and mitochondria are located in a centripetal position relative to the vascular bundle, except in some grass species of *Panicum* and *Eragrostis* (Ohsugi *et al.* 1982, Prendergast *et al.* 1986). The OAA formed in this metabolic sub-type is transformed into aspartate via aspartate

aminotransferase (EC 2.6.1.1.) in the cytoplasm of M cells (Fig. 1.3.), which is transported to BS cells and deaminated by aspartate aminotransferase in the mitochondria, forming OAA. The OAA is reduced to malate by NADP-MDH and malate is decarboxylated by NAD-ME in the mitochondria forming CO_2 and pyruvate. Pyruvate is then converted to alanine in the cytoplasm, which is shuttled to the M cells and transformed again in pyruvate (for revision see Kanai and Edwards 1999 and Arrabaça 2008).

NAD-ME subtype

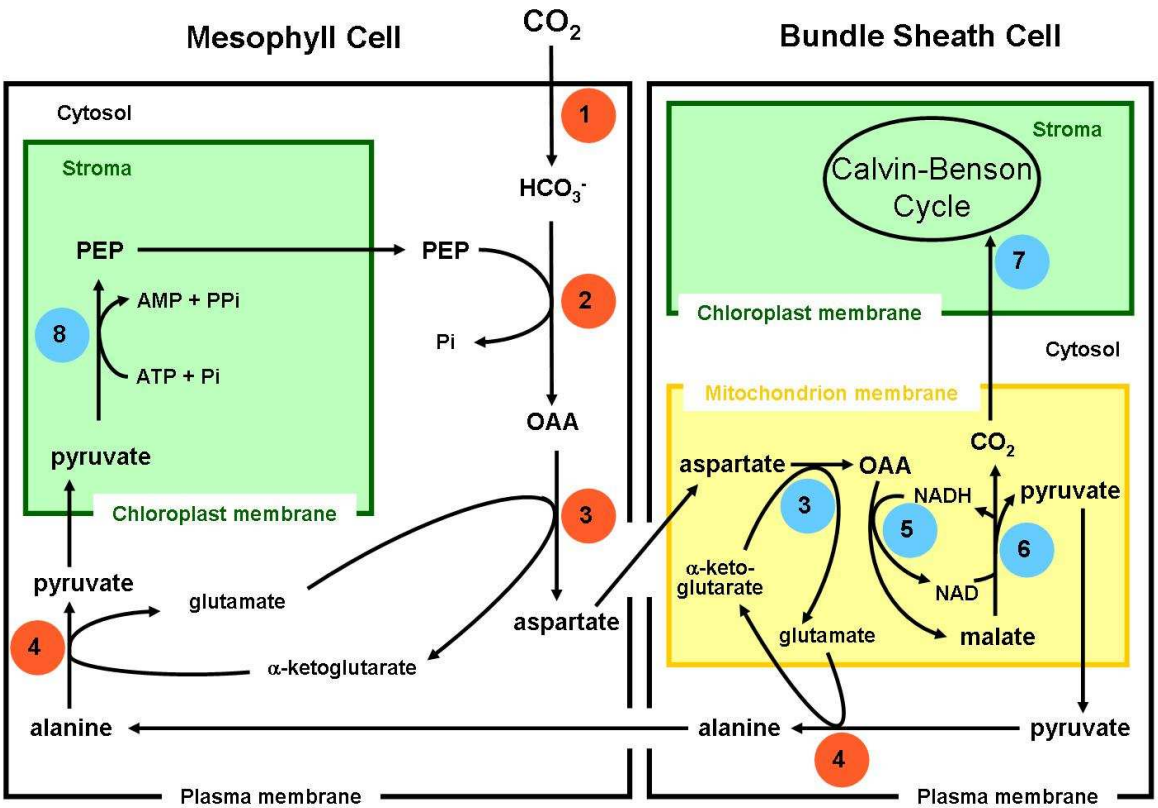

Figure 1.3. Simplified scheme of the key reactions and their location in the mesophyll and bundle sheath cells of a NAD-ME C_4 plant (Adapted from Kanai and Edwards 1999).
Compound abbreviations: AMP, adenosine 5'-monophosphate; ATP, adenosine 5'-triphosphate; HCO_3^-, bicarbonate ion; NAD, β-nicotinamide adenine dinucleotide; NADH, β-nicotinamide adenine dinucleotide, reduced; OAA, oxaloacetate; PEP, phospho*enol*pyruvate, Pi, inorganic phosphate; PPi, pyrophosphate.
Enzyme abbreviations: 1, carbonic anhydrase; 2, PEP carboxylase; 3, aspartate aminotransferase; 4, alanine aminotransferase; 5, NAD-malate dehydrogenase; 6, NAD-malic enzyme; 7, ribulose-1,5-bisphosphate carboxylase/oxygenase; 8, pyruvate phosphodikinase. Enzyme numbers inside red circles correspond to reactions occurring in the cytosol, while inside blue circles correspond to reactions occurring in the chloroplast and mitochondria.

As in the NAD-ME metabolic sub-type, chloroplasts of BS cells in the PEPCK metabolic sub-type have well-developed grana stacking. The BS chloroplasts are arranged evenly or in a centrifugal position in this C_4 sub-type. Although in this metabolic subtype the OAA is mainly transformed in aspartate by aspartate aminotransferase in the M cytoplasm, it may also be transformed in malate in the M chloroplasts (Fig. 1.4.). The aspartate is transported to BS cells where is deaminated in the cytoplasm to OAA, which is decarboxylated by PEPCK. The malate is transported to the BS mitochondria where is decarboxylated by NAD-ME. Thus, two decarboxylases may release CO_2 to BS chloroplasts. PEP formed in the cytoplasm of the BS cells by the decarboxylation of OAA returns directly to the M cytoplasm. Pyruvate formed in the NAD-ME decarboxylation may return to M chloroplasts through alanine, as in the NAD-ME metabolic sub-type (for revision see Kanai and Edwards 1999 and Arrabaça 2008).

1.4.3. Chilling sensitivity of C_4 plants

The most extensive natural C_4 biomes are the hot and dry savannas of the tropics and subtropics. However, plants with the C_4 photosynthetic metabolism can also dominate wet and warm temperate zones (for review see Sage *et al.* 1999). C_4 plants are also common in poor and saline coastal habitats from warm temperate to tropical regions.

In the whole world regions, C_4 grass representation is dependent upon latitude (e.g. Teeri and Stowe 1976, Hattersley 1983, Collins and Jones 1985, Taub 2000, Cabido *et al.* 2008), being more present at low latitudes. More than two-thirds of all grasses in the tropics and subtropics are C_4 and the grasses from arid regions at low latitudes belong almost exclusively to this photosynthetic pathway (for revision see Sage *et al.* 1999). However, there are some C_4 grasses that occur at high latitudes (> 60ºN), as species of the genera *Bouteloua*, *Miscanthus*, *Muhlenbergia* and *Spartina* (Long 1999, Sage *et al.* 1999). Species of the genera *Cynodon*, *Digitaria*, *Echinochloa*, *Eragrostis* and *Setaria* are often found between 50ºN and 60ºN (Sage *et al.* 1999). In addition, the contribution of C_4 species to local floras and vegetation shows a strong declining with increasing altitude (e.g. Teeri 1979 in Sage *et al.* 1999, Wentworth 1983, Cabido *et al.* 1997).

PEPCK subtype

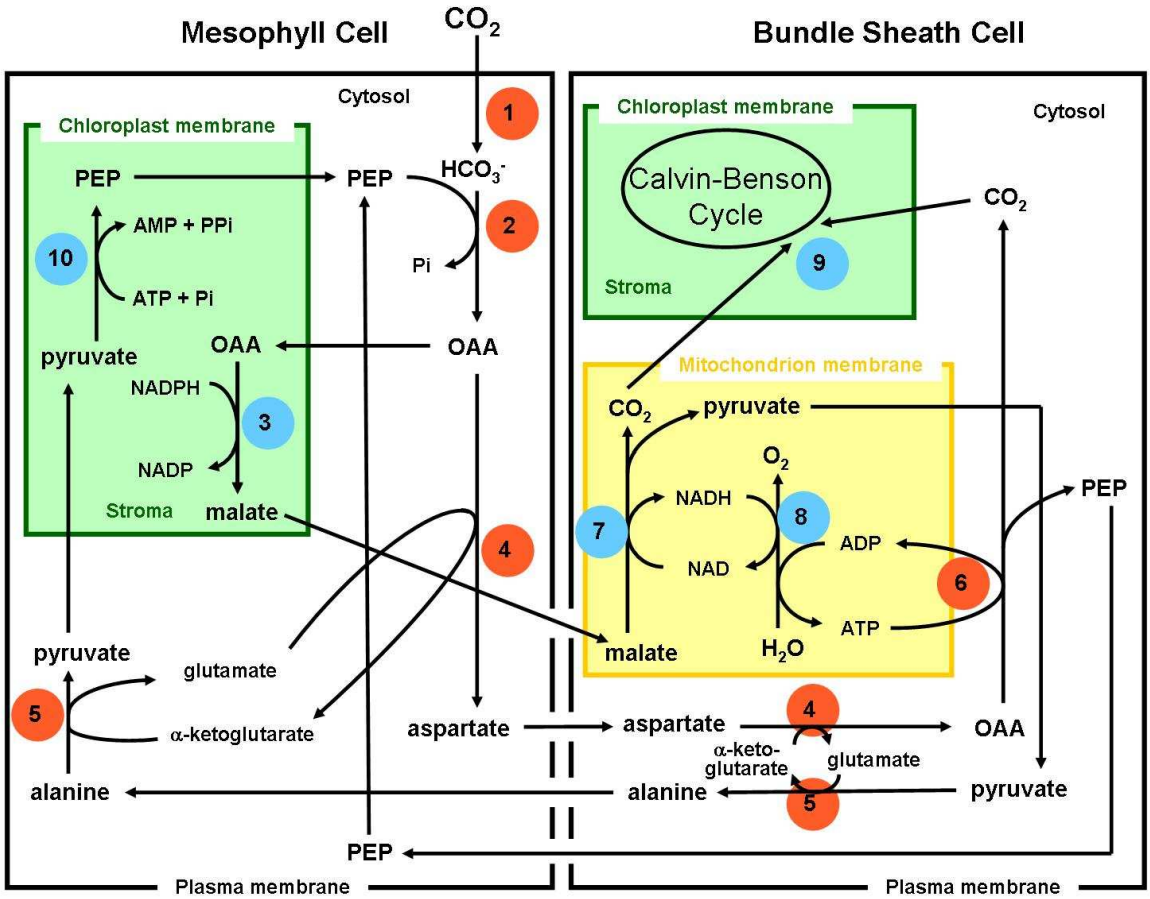

Figure 1.4. Simplified scheme of the key reactions and their location in the mesophyll and bundle sheath cells of a PEPCK C_4 plant (Adapted from Kanai and Edwards 1999).
Compound abbreviations: ADP, adenosine 5'-diphosphate AMP, adenosine 5'-monophosphate; ATP, adenosine 5'-triphosphate; HCO_3^-, bicarbonate ion; NAD, β-nicotinamide adenine dinucleotide; NADH, β-nicotinamide adenine dinucleotide, reduced; NADP, β-nicotinamide adenine dinucleotide phosphate; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced; OAA, oxaloacetate; PEP, phosphoenolpyruvate, Pi, inorganic phosphate; PPi, pyrophosphate.
Enzyme abbreviations: 1, carbonic anhydrase; 2, PEP carboxylase; 3, NADP-malate dehydrogenase; 4, aspartate aminotransferase; 5, alanine aminotransferase; 6, PEP carboxykinase; 7, NAD-malic enzyme; 9, ribulose-1,5-bisphosphate carboxylase/oxygenase; 10, pyruvate phosphodikinase. Enzyme numbers inside red circles correspond to reactions occurring in the cytosol, while inside blue circles correspond to reactions occurring in the chloroplast and mitochondria.
Other abbreviations: 8, electron transport respiratory chain.

Both the latitudinal and the altitudinal distribution of C_4 plants is highly correlated with temperature, decreasing as temperature decreases (for revision see Sage e*t al.* 1999). In North America, C_4 grasses, sedge and dicot representation is well correlated to July average and minimum temperature and number of days above 32ºC (Teeri and

Stowe 1976, Teeri *et al.* 1980). In Australia, increases in the mean annual temperature from 14º to 23ºC are well and positively correlated with C_4 representation (Hattersley 1983).

The rarity of most C_4 species in the cool temperate zones and their almost absence in the cold climate zones of the globe led to the hypothesis that there may be any step or steps in the C_4 photosynthetic pathway inherently unable to function efficiently at low temperatures. Both the cold liability of PEPC and PPdK, which have been showed to dissociate in *in vitro* studies at temperatures bellow 10ºC (Sugiyama 1973, Krall and Edwards 1993) may play a role in the almost absence of C_4 plants in colder climates. Furthermore, it has been suggested that the leading cold-sensitive step in C_4 photosynthesis is PEP regeneration by PPdK or PEPCK (Long 1983, Potvin *et al.* 1986, Leegood and Edwards 1996, Matsuba *et al.* 1997). Although some enzymes in the C_4 cycles may be cold labile, the presence of some C_4 species in cold climates (both at high altitudes and latitudes) indicates that the photosynthetic metabolism in these plants is not necessarily prone to failure in cold conditions, and thus chilling injury to photosynthetic enzymes may not be the ultimate explanation for the general absence of the C_4 syndrome in cold climates (Pittermann and Sage 2001).

An alternative explanation for the poor performance of C_4 photosynthesis at low temperature is related to the fact that Rubisco capacity, i.e. the capacity of fully activated Rubisco to consume RuBP under a given set of environmental conditions, limits the CO_2 assimilation at suboptimal temperatures (see for review Sage and Kubien 2007). Differences in the photosynthetic rates at low temperatures in the C_4 species *Atriplex lentiformis* grown at different temperatures were well correlated with changes in the maximum activity of Rubisco (Pearcy 1977). Furthermore, the rate of net CO_2 assimilation in the high latitude and altitude species *Bouteloua gracilis* (Pittermann and Sage 2000) and *Muhlenbergia montana* (Pittermann and Sage 2001) and in the high latitude *M. glomerata* (Kubien and Sage 2004) was equivalent to the maximum activity of Rubisco *in vitro* at suboptimal temperatures but much higher at temperatures above 25ºC. Recent work with anti-*Rbc*S (small sub-unit of Rubisco) *Flaveria bidentis* has shown that Rubisco capacity, more specifically the amount of the enzyme, is an important controlling step over the photosynthetic rate in C_4 plants at low temperatures (Kubien *et al*. 2003). Thus, the higher sensitivity of the C_4 photosynthetic metabolism to cold temperatures in relation to C_3 species may be related to the fact that Rubisco

content in a C_4 leaf is typically one-quarter to one-third of that of a C_3 plant and to the high thermal dependence of C_4 Rubisco maximal activity (Sage and Pearcy 2000). It has been shown that the chilling-tolerant C_4 grass *Miscanthus giganteus* maintains high photosynthetic rates when grown at low temperatures due to the ability to maintain high levels of Rubisco and PPdK proteins, while chilling-sensitive maize plants grown at 14ºC showed a decrease in the content of both of these enzymes relative to plants grown at 25ºC (Naidu *et al*. 2003).

1.4.4. Characterization of the C_4 species studied

The three C_4 grasses used in this study, *Paspalum dilatatum* Poir., *Cynodon dactylon* L. (Pers) and *Zoysia japonica* Steudel are classified as warm-season grasses (Hull 1996). P. *dilatatum* belong to the subfamily Panicoideae, tribe Paniceae, while *C. dactylon* and *Z. japonica* belong to the subfamily Chloridoideae, tribe Chlorideae and Zoysieae, respectively (Watson and Dallwitz 1992, Hull 1996). The three C_4 grass species belong to the three different metabolic subtypes (see Section 1.4.2). *P. dilatatum* is classified as a NADP-ME species (Usuda *et al.* 1984), *C. dactylon* as a NAD-ME species (Hatch and Kagawa 1974) and *Z. japonica* as a PEPCK species (Gutierrez *et al.* 1974). Additionally to the use of this three species as turfgrass throughout the world, *P. dilatatum* and *C. dactylon* are also important forage and cultivated pasture grass (Brown 1999). Furthermore, *C. dactylon* is also one of the world's most serious weeds (Jones 1985).

P. dilatatum, also known as Dallisgrass (Pinto da Silva 1969), is a tufted and perennial grass native from the Southeastern Brazil, Uruguay and Northern Argentina, but it also survives in many other tropical and subtropical countries (Pinto da Silva 1969, Jones 1985). It is currently well adapted to European countries, as Portugal, France and Spain, to South USA, among other regions of the world (Pinto da Silva 1969, Pitman *et al.* 1987). It is considered a semi-tolerant species to cold (Taylor *et al.* 1972, Cavaco *et al.* 2003), resisting to mild frosts (Rowley 1976). The chilling tolerance of *P. dilatatum* seems to correlate positively with significant alterations in the carbohydrate metabolism, namely the increase in the content of soluble sugars and starch (Taylor *et al.* 1972, Cavaco 2000). When *P. dilatatum* plants were subjected to a short-term chilling during the day and night periods, the relative growth and the

photosynthetic rates decreased (Taylor and Rowley 1971, Forde *et al.* 1975, Forde *et al.* 1977), as well as the content of malate (Taylor *et al.* 1972) and chlorophyll (Forde *et al.* 1975). Furthermore, the structure of M chloroplasts was altered (Forde *et al.* 1975).

C. dactylon, also known as Bermudagrass (Jones 1985), is a vigorous, stoliniferous and rhizomatous perennial grass native from Africa, but today is spread all over the tropical and subtropical regions of the world (Jones 1985, Brosnan and Deputy 2008). The stolons and rhizomes readily root at the nodes to form a dense turf (Jones 1985). A wide range of ecotypes and varieties are known, showing different characteristics of establishment and persistence (Andrews and Crofts 1979, Jones 1985). The ecotypes and varieties producing seeds are important weeds in many cultivated crops, while the sterile hybrids, reproduced by cutting, are valuable pastures and lawn grasses (Jones 1985). It is the most widely used turfgrass in lawn, golf course and other sport fields (Brosnan and Deputy 2008), and it is also used as forage for livestock (Starks *et al.* 2006). Although *C. dactylon* requirement of full sunlight for best performance (Teeg and Lane 2004, Brosnan and Deputy 2008), this species has been suggested as a potential energy crop for biofuel production (Boateng *et al.* 2007). Bermudagrasses have achieved their importance only in areas of relatively mild winters, however there are a few hardy varieties and hybrids that persist in areas with sub-zero temperatures (Anderson *et al.* 2007). Several studies have been performed to evaluate the freezing tolerance of Bermudagrass (e.g. Anderson *et al.* 1993, Anderson and Taliaferro 1995, Anderson and Taliaferro 2002, Anderson *et al.* 2002, Anderson *et al.* 2003). Studies related to the cold acclimation of Bermudagrasses were also done, focusing the effect of cold in fatty acid composition (Samala *et al.* 1998, Cyril *et al.* 2001*b*) and leaf metabolism (Zhang *et al.* 2006*a*). Cold acclimation decreases the sensitivity of Bermudagrass to freezing stress (Zhang *et al.* 2006*a*).

Z. japonica, also known Zoysiagrass, Japanese or Korean lawn grass (Duble 2002), is a rhizomatous perennial grass native from Asia, which was introduced in North America at the beginning of the twenty century (Deputy and Hensley 1999). Zoysiagrasses are shade tolerant and perform well in lawns, being widely used in golf courses and other sport fields (Deputy and Hensley 1999). As Bermudagrasses, Zoysiagrasses are fertile, seedling species with improved selections, also presenting sterile hybrids that must be propagated vegetatively (Deputy and Hensley 1999). It spreads by underground rhizomes, resulting in a thick carpet of turf that tends to choke

out all other grasses and weeds (Deputy and Hensley 1999). Species with different sensitivities to cold are found in Zoysiagrass depending on the variety or hybrid studied (Patton and Reicher 2007). The physiological basis for the differences in cold hardiness and freeze tolerance in this species genotypes have been studied (e.g. Rogers *et al.* 1975, Rogers *et al.* 1977, Patton *et al.* 2007*a*, Patton *et al.* 2007*b*), showing the importance of dehydrins, proline, and glucose content for species freezing tolerance.

1.5. Aims of this Thesis

The expected increase in temperature due to climate changes (IPCC 2007) may enhance the proportion of C_4 species in non-tropical and subtropical regions. However, low temperatures at night before a mild day are still expected increasing the damages due to cold (Bracale and Coraggio 2003), greatly affecting the production of C_4 plants. Thereby, the study of the effects of dark-chilling on subsequent photosynthetic metabolism at warm temperatures is of particular interest in C_4 plants, especially in those less sensitive to cold which may be rapidly successful in non-tropical and subtropical regions. Although the plants in these study are C_4, and thus more susceptible to cold than C_3 plants, the cultivars used are considered less cold sensitive than other C_4 species as maize and sorghum.

The scarcity of reports studying the effects of dark-chilling on the subsequent warm light period metabolism in C_4 plants, namely from different metabolic sub-types, shows the pertinence of the present study in order to get a better understanding of the physiological and metabolic changes caused by dark-chilling in these species. One of the main aims of this study was to understand if the changes in photosynthesis and electron transport after the low night temperature were dependent on the light intensity on the subsequent warm day. In addition, we aimed to study the effects of short-term dark-chilling stress *per se*, i.e. without the simultaneous effect of light intensity, on the leaf fatty acid composition and the photosynthetic metabolism. Finally, this research was conducted with the objective of understanding if the response of photosynthesis on the whole leaf and on each leaf surface separately was altered after a short-term dark-chilling and if it was differently affected under adaxial or abaxial illumination. This final study was only performed in *P. dilatatum* belonging to the subtype NADP-ME, the

most representative group of C_4 species. To achieve this goal, the characterization of the whole leaf and each leaf surface photosynthetic response to light orientation was also performed under control conditions.

To attain these objectives we started this study by comparing the effects of one and two nights of chilling stress on the leaf parameters and on the photosynthetic rate in *P. dilatatum*, *C. dactylon* and *Z. japonica* in order to choose the number of night-cold periods to apply to further studies (Chapter 2). Gas-exchange and chlorophyll *a* fluorescence analysis were performed on the subsequent warm light period after one night-chilling at low, medium and high light intensities (Chapter 3). The metabolic changes were studied at the lowest light intensity after one night-chilling (Chapter 4), assaying the fatty acid composition, membrane permeability, lipid peroxidation, carbohydrate and protein content and carboxylating enzymes activity. In order to characterize the photosynthetic rate and stomatal regulation on the whole leaf and on the adaxial and abaxial leaf surfaces separately in *P. dilatatum* plants, CO_2- and light-response curves of photosynthesis under adaxial and abaxial illumination were performed (Chapter 5). This was accompanied by immunological, microscopy and leaf optical studies on each leaf surface. The dark-chilling effect of one and two nights was observed on the whole leaf and on each surface separately with the leaf illuminated from the adaxial or the abaxial surface (Chapter 6). In this Chapter, photosynthetic models were applied to the CO_2- and light-response curves of whole leaf photosynthesis allowing the determination of several parameters.

With the results obtained in this study we hope to contribute to the general knowledge of the behaviour of C_4 plants on the subsequent warm day after a dark-chilling stress, and to address the question whether the changes observed in the C_4 species metabolism are similar to those usually described in the literature for their C_3 counterparts. We also hope to contribute for the understanding of the different adaxial and abaxial photosynthesis regulation of the NADP-ME species *P. dilatatum*, as recently described for the NADP-ME maize species (Driscoll *et al.* 2006), and how this differential regulation is affected by dark-chilling.

1.6. References

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Chapter 2.

Leaf parameters and photosynthetic rate in C_4 gramineae from different metabolic sub-types following one and two nights at low temperature.

The results presented in this Chapter were obtained by Ana Sofia Soares at the laboratory of the Secção de Fisiologia e Bioquímica Vegetais, Departmento de Biologia Vegetal, Faculdade de Ciências, Universidade de Lisboa.

A manuscript to submit for publication in an international peer-reviewed journal is being prepared with part of the results presented in this Chapter, together with part of those presented in Chapter 3 and Chapter 4. These results were also presented in the following conferences:

- Soares AS, Bernardes da Silva A, Marques da Silva J, Arrabaça MC (2005) Photosynthetic response of C_4 grasses with different decarboxylating mechanisms to short-term chilling stress. In: Comparative Biochemistry and Physiology, Society for Experimental Biology (SEB) Annual Main Meeting 2005 Abstracts, Universitat Autónoma de Barcelona, Barcelona, Espanha, Volume 141/A, Number 3/Suppl., S275-S275 – Oral Presentation.

- Soares AS, Bernardes da Silva A, Marques da Silva J, Arrabaça MC (2005) Short-term dark-chilling stress does not impair the photosynthetic parameters in the C_4 gramineae *Cynodon dactylon*. In: Section 2: Light and Temperature Effects on Photosynthesis. Photosynthesis and Stress, Central-European Conference, Brno, República Checa – Oral Presentation.

2. Leaf parameters and photosynthetic rate in C_4 gramineae from different metabolic sub-types following one and two nights at low temperature.

2.1. Abstract

In this chapter we have investigated the effects of a short-term low night temperature (5±3ºC) on several leaf parameters and on the photosynthetic rate in the subsequent light period at 25ºC in C_4 grasses of the three metabolic sub-types, *Paspalum dilatatum* Poiret (NADP-ME), *Cynodon dactylon* (L.) Pers (NAD-ME) and *Zoysia japonica* Steudel (PEPCK). The plants were subjected to one or two nights of chilling in order to decide which stress should be applied in the further studies. One night-chilling did not affect the leaf relative water content (*RWC*) and the area/fresh weight ratio in any of the plants studied. However, it has increased the dry/fresh weight ratio (*DW/FW ratio*) and decreased the specific leaf area in *P. dilatatum* and *C. dactylon*. Net photosynthetic rate (*A*) and stomatal conductance to water vapour (*gs*) decreased greatly in *Z. japonica*, moderately in *P. dilatatum* and were not affected in *C. dactylon* after one night-chilling. The second night of chilling led to a decrease in the *RWC* and an increase in the *DW/FW ratio* in *Z. japonica*, as well as a recovery of some leaf parameters to control values in *P. dilatatum* and *C. dactylon*. In addition, *A* and *gs* showed either similar or a tendency to recover to the control values in *P. dilatatum* and *Z. japonica*. Since one night-chilling led to more pronounced effects, we have chosen this stress to further studies on the following chapters.

Keywords: one night-chilling, two nights-chilling, C_4 plants, leaf relative water content, specific leaf area, photosynthesis, stomatal conductance.

2.2. Introduction

The responses of plants to a low temperature during the day and night periods have been widely studied, both in relation to long- and short-term chilling (e.g. Taylor and

Rowley 1971, Kingston-Smith *et al.* 1997, Cavaco *et al.* 2003). However, there are a few studies, mainly concerning C_3 plants, which focus the effects of night-chilling on plant metabolism and photosynthesis on the subsequent warm light period.

Dark-chilling has been shown to decrease the leaf relative water content (*RWC*) in C_3 plants immediately after the first night (Bauer *et al.* 1985, Flexas *et al.* 1999, Allen *et al.* 2000, van Heerden *et al.* 2003*a*). Furthermore, the authors showed that this decrease just occurs when the roots were chilled, contrasting to results obtained with low temperature during both light and night periods where a decrease in *RWC* may also occur when only the shoot is chilled (Schultz and Matthews 1988, Lovisolo and Schubert 1998). The decrease in *RWC* may result from the negative effect of chilling on root conductivity, water absorption and translocation (Hällgren and Öquist 1990, Rab and Saltveith 1996 and references therein). At our best knowledge, the effect of dark-chilling on other leaf parameters was never reported, both in C_3 and C_4 plants. However, low temperatures may alter the carbohydrate metabolism of leaves (Potvin e*t al.* 1984), affecting leaf density, what may induce changes in the specific leaf area.

Chloroplast structure is the first and the most severely impaired by a short-term low temperature (for review see Kratsch and Wise 2000). Thus, it is not surprising that the best characterized response of chilling-sensitive C_3 plants to dark-chilling is a decrease in photosynthesis on the subsequent warm light period, as observed in cucumber (Jun *et al.* 2001, Zhou *et al.* 2004), soybean (e.g. van Heerden *et al.* 2003*b*, Strauss *et al.* 2007), coffee (Bauer *et al.* 1985), grapevine (e.g. Flexas *et al.* 1999) and tree species (Allen *et al.* 2000, Feng and Cao 2005). The few studies that exist in C_4 plants show that photosynthesis decreased after low night temperatures, as for example in maize (Long *et al.* 1983) and other species (Ludlow and Wilson 1971, Pasternak and Wilson 1972, Ivory and Whiteman 1978, Pittermann and Sage 2001). Nevertheless, the chilling effects on photosynthesis may not occur immediately after the first night of chilling depending on the degree of species sensitivity (van Heerden *et al.* 2003*b*, Feng and Cao 2005). Furthermore, photosynthesis showed a total or a partial recovery to control values in some species after the second or more nights of chilling (Bauer *et al.* 1985, Flexas *et al.* 1999, van Heerden *et al.* 2003*b*). Dark-chilling limitations to photosynthesis, largely described for C_3 plants, involve both stomatal and non-stomatal factors (Jun *et al.* 2001, van Heerden *et al.* 2003*b*, Zhou *et al.* 2004).

There is a lack of information in the literature on the C_4 plant responses after a dark-chilling. To study the effects of this stress on plant metabolism and photosynthesis, we first have chosen the stress period to which the plants were subjected. With that purpose we have observed the effects of one and two nights of chilling (5±3ºC) on some leaf parameters and on the photosynthetic rate of three C_4 warm-grasses of different metabolic sub-types, *Paspalum dilatatum* cv. Raki (NADP-malic enzyme, NADP-ME), *Cynodon dactylon* var. Shangri-La (NAD-malic enzyme, NAD-ME) and *Zoysia japonica* "Jacklin Sunrise Brand" (phospho*enol*pyruvate carboxykinase, PEPCK).

2.3. Material and Methods

2.3.1. Plant material and growth conditions

Seeds of *Paspalum dilatatum* Poiret cv. Raki (NADP-malic enzyme, NADP-ME), *Cynodon dactylon* (L.) Pers var. Shangri-La (NAD-malic enzyme, NAD-ME) and *Zoysia japonica* Steudel "Jacklin Sunrise Brand" (phospho*enol*pyruvate carboxykinase, PEPCK) plants were sown as described by Carmo-Silva, Soares *et al.* (2007). Plants were hydroponically grown in a controlled environment chamber under a photosynthetic photon flux density (PPFD) of approximately 250-300 μmol m^{-2} s^{-1}, a photoperiod of 16 h and a temperature of 25/18ºC (day/night) (Fig. 2.1.). Light was provided by Halolux Ceram 64476KL incandescent lamps, Fluora L58W/77 and L30W/77 fluorescent lamps and Powerstar HQI-BT-400W/D sodium vapour lamps, all from Osram (Germany). After germination plants were watered with a KB Universal Nutrient Solution (Scotts France SAS, Lyon, France), supplied with additional Fe-EDTA, $MgSO_4$ and NaCl. The final concentration of the nutrients in the solution was 18 mM N, 5.8 mM P, 5.4 mM K, 1.5 mM Mg, 1.5 mM S, 0.6 mM Na, 122.5 μM Fe, 32 μM B, 11 μM Cu, 6.3 μM Mn, 1 μM Zn and 0.3 μM Mo, following the Hewitt (1966) nutrient solution with some modifications. This solution was replenished as needed and completely renewed once a week.

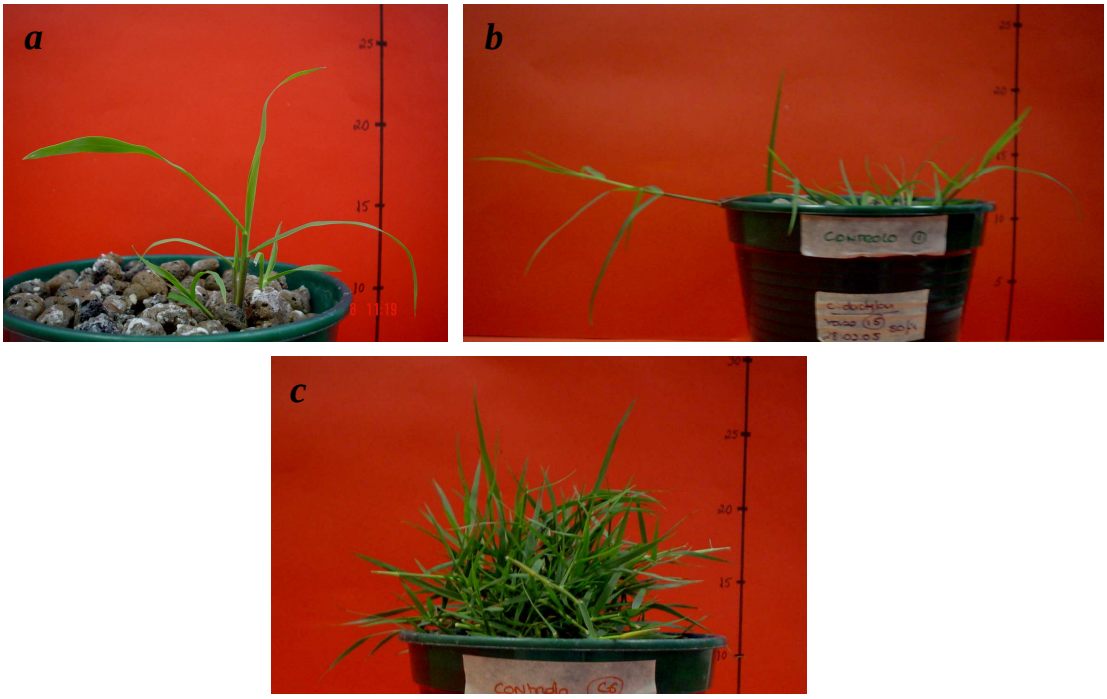

Figure 2.1. The three C_4 species studied, *Paspalum dilatatum* (***a***), *Cynodon dactylon* (***b***) and *Zoysia japonica* (***c***). Photographs correspond to control plants grown for 6 weeks after sowing for *P. dilatatum* and *C. dactylon* and 12 weeks for *Z. japonica*.

2.3.2. Dark-chilling stress imposition

Dark-chilling stress was imposed 6 weeks after sowing for *P. dilatatum* and *C. dactylon*, and 12 weeks for *Z. japonica*, due to the slow growing rate of the latter species. The stress was imposed for one or two consecutive nights by transferring the plants from the growth chamber (25/18ºC, day/night) to a cold chamber (5±3ºC) at the beginning of each night period. After each night-chilling the plants were returned to the growth chamber and leaf parameters samples were collected from one to two hours after the beginning of the light period. Gas-exchange measurements were performed from one to five hours after the plants transference. Values obtained in the above assays were constant in the first hours of the light period. All the assays were performed on the middle and widest part of fully expanded leaves.

2.3.3. Leaf parameters determination

Leaf fresh, turgid and dry weights (*FW*, *TW* and *DW*, respectively) and leaf area (portable leaf area meter CI-202, CID Inc., Camas, Washington, USA) were determined for control, one and two nights-chilled plants. The leaf relative water content (*RWC*) of each sample was determined according to Catsky (1960). The specific leaf area (*SLA*, area to dry weight ratio) was also determined.

Each value presented is the mean of eight plants *per* treatment.

2.3.4. Gas-exchange measurements

Photosynthetic gas-exchange measurements were performed using an LCpro+ portable infra red gas analyser (ADC BioScientific Ltd., Hoddesdon, Hertfordshire, UK) fitted with a broad leaf Parkinson chamber. Values of net CO_2 assimilation rate (*A*) and stomatal conductance to water vapour (*gs*) were obtained for control, one and two nights-chilled plants. Steady-state gas-exchange measurements were obtained after 15 min at a leaf temperature of 25ºC, 350 μL L^{-1} CO_2 concentration, relative humidity of 40 to 50% and a PPFD of 500 μmol m^{-2} s^{-1} provided by an external led light unit (ADC BioScientific Ltd., Hoddesdon, Hertfordshire, UK) that allows a cool and well distributed illumination. After each measurement, the area of the enclosed sample was determined.

Each value is the mean of eight plants *per* treatment.

2.3.5. Statistical analysis

The data were statistically analysed using parametric tests at a stringency of $P<0.05$ with the program Statistical Package for Social Sciences (SPSS) 15.0, 2006 (SPSS Inc., Chicago, Illinois, USA). The significance of variation in the mean values for the leaf parameters and gas-exchange measurements between control, one and two nights-chilled plants of each species was determined using an ANOVA test followed by a Tukey HSD test. All the analyses were performed for each species separately, what was evidenced by the use of small letters and apostrophes in the figures and tables.

2.4. Results

2.4.1. Leaf parameters

There was no visible effect of low night-temperature on plants, with *P. dilatatum*, *C. dactylon* and *Z. japonica* control and dark-chilled plants showing a similar phenotype (Fig. 2.1.). The mean *RWC* value in the control plants (18ºC night temperature) of the three species was always above 90%. *Paspalum dilatatum* and *C. dactylon RWC* was not affected on the subsequent warm light period after one or two consecutive nights of cold (5±3ºC) imposition (Fig. 2.2.*a*). However, in *Z. japonica*, the *RWC* decreased slightly (around 5%) after two consecutive nights of stress.

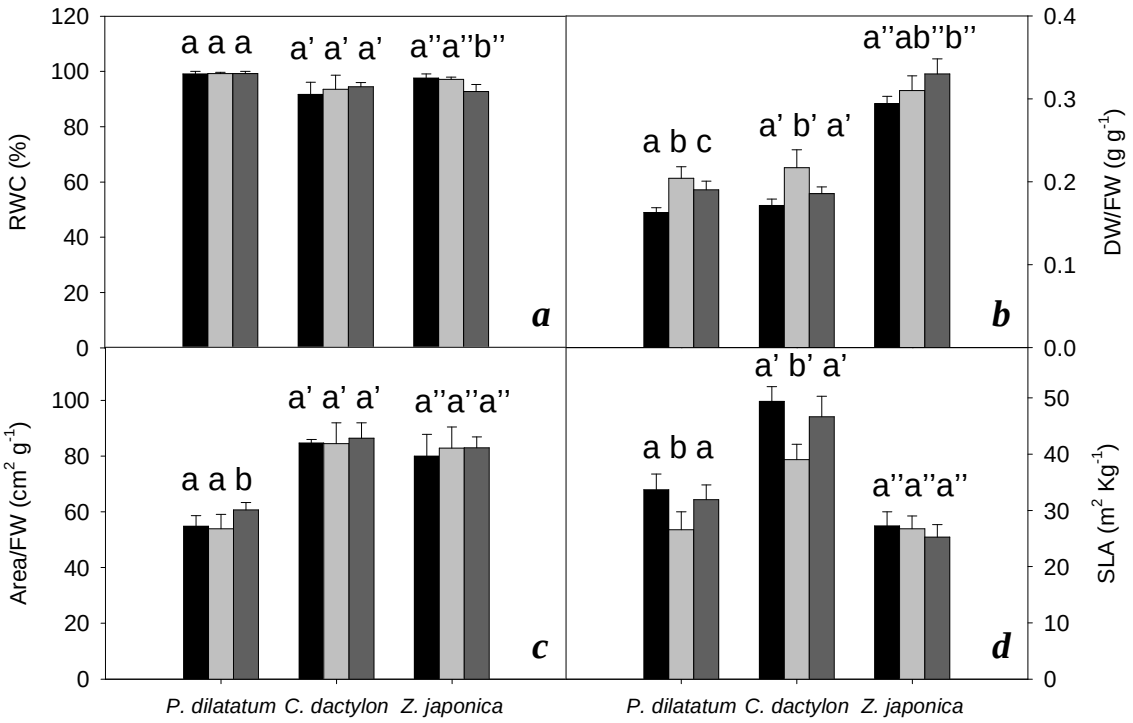

Figure 2.2. Leaf parameters of control (black bars), one (grey bars) and two (dark grey bars) nights-chilled plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. Data for leaf relative water content (*RWC*, ***a***), dry to fresh weight ratio (*DW/FW*, ***b***), area to fresh weight ratio (*Area/FW*, ***c***) and specific leaf area (*SLA*, ***d***) correspond to the mean ± SD of eight plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at P<0.05.

In *Z. japonica* control plants the dry to fresh weight ratio (*DW/FW ratio*) was almost twice of that of control *P. dilatatum* and *C. dactylon* (Fig. 2.2.*b*). One night of chilling stress did not affect this *ratio* in *Z. japonica*, but increased it 25% and 30% in *P. dilatatum* and *C. dactylon* plants, respectively. After two nights of chilling the *DW/FW ratio* was similar to that of control in *C. dactylon* plants, while in *P. dilatatum* an increase was observed, although lower than after one night of chilling. In *Z. japonica* an increase in this ratio was observed after two nights-chilling (14% increase compared with control values). The higher area to fresh weight ratio (*Area/FW ratio*) was found in *C. dactylon* and *Z. japonica* and was not affected by the night-chilling (Fig. 2.2.*c*). In *P. dilatatum* this ratio increased 12% only in plants subjected to two consecutive nights of chilling.

The *SLA* in the control plants was higher in *C. dactylon* and lower in *Z. japonica* (Fig. 2.2.*d*). In *P. dilatatum* and *C. dactylon*, the *SLA* decreased *circa* 20% in plants subjected to one night of chilling but presented the same value as control plants after two consecutive nights of cold. On the contrary, in *Z. japonica* the *SLA* was not affected by one or two nights of chilling.

2.4.2. Gas-exchange parameters

In the control plants *A* and *gs* were lower in *C. dactylon* (Fig. 2.3.). Both parameters were not affected by the dark-chilling stress in this species. However, *A* and *gs* decreased similarly after one night-chilling, around 25% and 45% in *P. dilatatum* and *Z. japonica*, respectively. When these plants were subjected to the second consecutive night of cold *A* showed similar values to those of control in *P. dilatatum*, while in *Z. japonica* it showed a decrease, although lower than after one night-chilling (Fig. 2.3.*a*). The *gs* values in both *P. dilatatum* and *Z. japonica* were similar in one and two nights-chilled plants (Fig. 2.3.*b*).

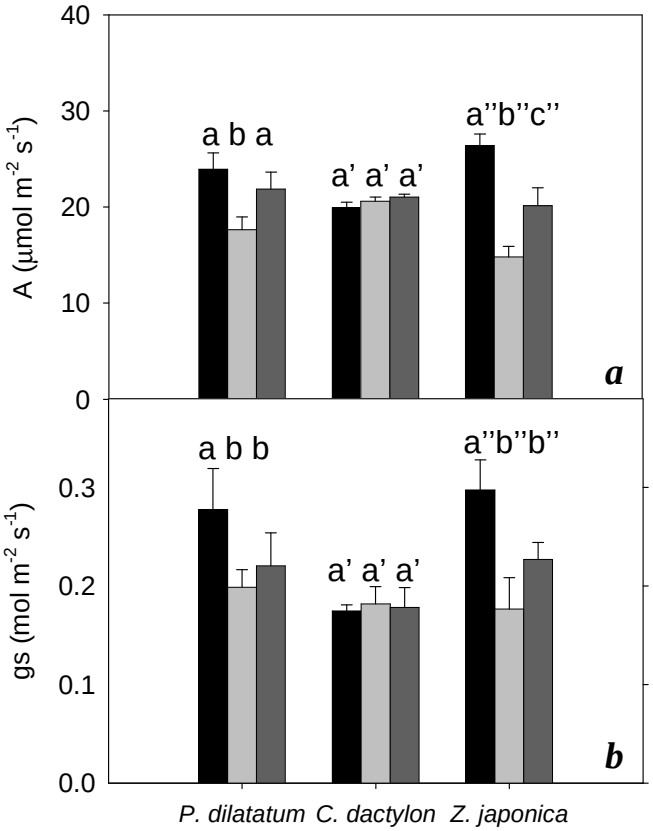

Figure 2.3. Gas-exchange parameters of control (black bars), one (grey bars) and two (dark grey bars) nights-chilled plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. Measurements of net CO_2 assimilation rate (*A*, ***a***) and stomatal conductance to water vapour (*gs*, ***b***) were performed under a CO_2 concentration of 350 µL L^{-1}, at 25ºC and at a PPFD of 500 µmol m^{-2} s^{-1}. Data are mean ± SD of eight plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at $P<0.05$.

2.5. Discussion

2.5.1. In general one night-chilling decreased the specific leaf area and increased the DW/FW ratio

The leaf parameters measured on the following warm day after dark-chilling show different responses depending on the number of consecutive nights the plant is chilled, generally with a total or partial recovery after two nights-chilling.

It is well known that prolonged root chilling decreases water uptake (for review see Allen and Ort 2001) due to a decreased water conductivity of roots at low temperature (Rab and Saltveith 1996 and references therein), inducing plant water stress. The

general non-variation and high *RWC* values obtained after one and two nights-chilling in the three C_4 species under study (Fig. 2.2.*a*) may indicate that low night temperature have not induced a water stress condition. van Heerden *et al.* (2003*a*) showed that in soybean plants the *RWC* decreased greatly after the first chilling-night and was maintained low thereafter. However, this behaviour just occurred if the whole plant was exposed to the dark-chilling episode, suggesting that the water stress effect on soybean leaves only occurs if roots were chilled. Mango (Allen *et al.* 2000) and coffee (Bauer *et al.* 1985) plants chilled for one night at the shoot level also show no effect of low night temperature on *RWC*, but chilling of whole grapevine plants showed a decrease in this parameter (Flexas *et al.* 1999). The different *RWC* response to dark-chilling of the whole plant found between the three C_4 species under study and the C_3 plants may be related to the higher competitiveness of the former species under water deficit conditions (Sage *et al.* 1999).

The decrease in *P. dilatatum* and *C. dactylon SLA* after one night-chilling may result from an increase in their leaf density (reflected by *DW/FW ratio*) since the dark-chilling treatment generally did not affect the leaf thickness (approximated by *Area/FW ratio*) (Fig. 2.2.,*b,c,d*). On the contrary, a long-term exposure to 4ºC during day and night of *Atriplex vesicaria* (also native from warm areas) showed a much thicker leaf (Caldwell *et al.* 1977). However, the same authors found no change in the leaf thickness in *Atriplex confertifolia* plants, native from cold areas. A low *SLA* value often results from the accumulation of secondary compounds like tannins, phenols and the quantitatively most important lignins and starch (for a review see Konings 1989). In accordance with our present results, cold acclimated *P. dilatatum* plants showed a decrease in the *SLA* (Cavaco *et al.* 2003), which was partially associated with an increase in starch content (Cavaco 2000). However, maize plants (a C_4 species) subjected to a short period of cold presented no changes in this parameter (Irigoyen *et al.* 1996). The above results show that the effects of chilling on the leaf parameters depend on the type and duration of the stress.

The non-variation of *SLA* after two consecutive nights of chilling in *P. dilatatum* and *C. dactylon*, due to a small or null variation of *DW/FW ratio* in comparison with control plants, showed that these two gramineae rapidly changed their metabolism in response to the extended chilling in order to achieve a new homeostatic equilibrium. On the contrary, *Z. japonica* leaf density presented a small increase only in the second night

of chilling, however with no variation in the *SLA*, which indicates that the metabolism of this species may be less sensitive to a short-term dark-chilling.

2.5.2. Photosynthesis was affected by short-term dark-chilling only in *P. dilatatum* and *Z. japonica*

Photosynthesis is known to decrease after one or several nights of chilling, both in C_3 plants (e.g. Bauer *et al.* 1985, Flexas *et al.* 1999, van Heerden *et al.* 2003*a*, Feng and Cao 2005) and in C_4 species (Ludlow and Wilson 1971, Pasternak and Wilson 1972, Ivory and Whiteman 1978, Long *et al.* 1983, Pittermann and Sage 2001). Our results show that photosynthesis decreased after one night of chilling in *Z. japonica* and *P. dilatatum* (Fig. 2.3.*a*), as also shown for the C_4 NAD-ME species *Muhlenbergia montana* (Nutt.) (Pittermann and Sage 2001). However, the CO_2 assimilation rate was maintained after the dark-chilling treatment (one or two consecutive nights) in the NAD-ME species *C. dactylon* (Fig. 2.3.*a*). In the soybean Maple Arrow genotype, less sensitive to cold, the photosynthesis was also maintained after one night-chilling, but decreased greatly in the second consecutive night of chilling (van Heerden *et al.* 2003*b*). Feng and Cao (2005) just found a decrease in photosynthesis on the fourth consecutive chilling night in the tropical tree *Calophyllum polyanthum*. In *P. dilatatum* photosynthesis recovered to control values after the second night of chilling, while in *Z. japonica* it recovered slightly but still presented a lower rate in comparison with unchilled plants. Similar responses to that of *P. dilatatum* were observed in coffee plants (Bauer *et al.* 1985), while in most species the recovery of photosynthesis was not total as in *Z. japonica* (e.g. van Heerden *et al.* 2003*b*, Feng and Cao 2005).

As for photosynthesis, *C. dactylon* plants did not show any change in *gs* (Fig. 2.3.*b*), as observed in soybean Maple Arrow genotype (van Heerden *et al.* 2003*b*) and *C. polyanthum* (Feng and Cao 2005) after one night-chilling. In *P. dilatatum* and *Z. japonica gs* changed similarly to photosynthesis after one nigh-chilling (Fig. 2.3.*b*), which may indicate stomatal limitations to photosynthesis in response to stress, as also observed in the literature for C_4 plants (Pasternak and Wilson 1972, Ivory and Whiteman 1978). However, the presence of non-stomatal limitations to photosynthesis may not be excluded, since it is known that a dark-chilling stress restrict the activity of the photosynthetic enzymes (e.g. Jones *et al.* 1998, van Heerden *et al.* 2003*b*) and

impairs the circadian regulation of photosynthetic gene transcription (Martino-Catt and Ort 1992). The fact that in *P. dilatatum* and *Z. japonica* the *gs* was more affected than *A* after the second night of chilling may suggest that stomatal opening is more sensitive to chilling than the photosynthetic rate.

In accordance with literature for C_3 plants (van Heerden *et al.* 2003*b*, van Heerden *et al.* 2004, Feng and Cao 2005), the effect of dark-chilling on the photosynthetic rate depends on the number of nights the plants were chilled and on the sensitivity of plants to this stress, with photosynthesis being greatly affected in *Z. japonica*, moderately in *P. dilatatum*, but not affected in *C. dactylon*.

2.6. Conclusion

The effect of dark-chilling on the leaf parameters and photosynthetic rate in these three C_4 species depend on the number of nights the plants were chilled and on the species sensitivity to the stress. The most affected leaf parameters after one night-chilling were the *DW/FW ratio* and the *SLA*, although only in *P. dilatatum* and *C. dactylon*. In contrast, photosynthesis was more affected in *Z. japonica*, followed by *P. dilatatum*. Since after two consecutive nights of chilling most of the studied parameters tend to show values similar to those of control it was decided in future studies to access the effects of one night-chilling in the photosynthesis and metabolism of the C_4 species *P. dilatatum*, *C. dactylon* and *Z. japonica*.

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Chapter 3.
Photosynthesis at three light intensities of C_4 gramineae of different metabolic sub-types after one night-chilling.

The results presented in this Chapter were obtained by Ana Sofia Soares at the laboratory of the Secção de Fisiologia e Bioquímica Vegetais, Departmento de Biologia Vegetal, Faculdade de Ciências, Universidade de Lisboa.

A manuscript to submit for publication in an international peer-reviewed journal is being prepared with part of the results presented in this Chapter, together with part of those presented in Chapter 2 and Chapter 4. These results were also presented in the following conference:

- Soares AS, Marques da Silva J, Bernardes da Silva A, Arrabaça MC (2005) Short-term dark chilling stress: Photosynthetic light response curves of the gramineae *Paspalum dilatatum*. In: Sessão 10: Fotossíntese e Respiração, Livro de Sumários do IX Congresso Luso-Espanhol de Fisiologia Vegetal e XIV Reunião da Sociedade Espanhola de Fisiologia Vegetal, Évora, Portugal – Panel Presentation.

3. Photosynthesis at three light intensities of C_4 gramineae of different metabolic sub-types after one night-chilling.

3.1. Abstract

In this Chapter the effects of one night of chilling (5±3ºC) on the subsequent photosynthetic rate and electron transport chain at three light intensities (200, 530 and 1300 μmol m^{-2} s^{-1}) were studied in three C_4 grasses, *Paspalum dilatatum* Poiret (NADP-ME), *Cynodon dactylon* (L.) Pers (NAD-ME) and *Zoysia japonica* Steudel (PEPCK). Results showed that the damages caused by one night-chilling on photosynthetic rate and electron transport depend on the light intensity on the subsequent warm period (25ºC). One night-chilling did not affect photosynthetic rate at low light intensity in any of the species studied. On the contrary, moderate and high light intensities decreased photosynthesis and chlorophyll *a* fluorescence parameters in *P. dilatatum* and *Z. japonica* dark-chilled plants, but higher light intensities were required to decrease photosynthesis in *C. dactylon* dark-chilled plants. Stomatal limitations to photosynthesis seem to be predominant, but electron transport limitations may also exist in *P. dilatatum* dark-chilled plants at the higher light intensity. We conclude that the effects on photosynthesis after one night-chilling are small at low light intensity, but they alter the capacity of plants to face an increase in light, although to a lesser extent in the NAD-ME species *C. dactylon*.

Keywords: chlorophyll *a* fluorescence parameters, C_4 photosynthesis, one night-chilling, stomatal conductance.

3.2. Introduction

It has been referred that the extent of photosynthesis inhibition is smaller if plants were only chilled during the night period (Allen and Ort 2001), however the mechanisms involved are not yet clearly understood. The majority of the night-chilling studies on photosynthesis have been performed in C_3 species (e.g. Flexas *et al.* 1999,

van Heerden *et al.* 2003*a*, Bertamini *et al.* 2005, Feng and Cao 2005). Studies concerning the effects of low night temperature in the C_4 photosynthetic metabolism are scarce (Ludlow and Wilson 1971, Pasternak and Wilson 1972, Ivory and Whiteman 1978, Pittermann and Sage 2001) and mainly report the effects of dark-chilling in the photosynthetic rate and stomatal conductance. In C_3 plants the negative effect of dark-chilling on the photosynthetic rate depends on the duration of the stress and on the species sensitivity to cold (e.g. van Heerden and Krüger 2002, van Heerden *et al.* 2003*a*, Zhou *et al.* 2004). In addition, Feng and Cao (2005) observed that they also depend on the light intensity on the following warm day, with damages increasing with light intensity.

Stomatal limitations have been found to occur in C_3 and C_4 dark-chilled plants, contributing to the decrease in photosynthetic rates (e.g. Pasternak and Wilson 1972, Martin *et al.* 1981, Bauer *et al.* 1985, van Heerden *et al.* 2003*a*, Feng and Cao 2005). Non-stomatal effects also contribute to the decrease in CO_2 assimilation rate after a dark-chilling in C_3 plants. The performance of the electron transport lowered in these plants after dark-chilling, namely at the PSII reaction centres level (Shen *et al.* 1990, van Heerden *et al.* 2003*b*, Bertamini *et al.* 2006, Strauss *et al.* 2007). Non-photochemical quenching coefficient increased after dark-chilling (Flexas *et al.* 1999, Feng and Cao 2005), suggesting the participation of the xantophyll cycle in the excess light dissipation. Although there is no reason to think that the C_4 plants will behave differently than their C_3 counterparts, as far as we know any study has ever evaluate the effect of dark-chilling on the C_4 chloroplasts electron transport chain.

In this Chapter the three C_4 gramineae from the different metabolic subtypes (*Paspalum dilatatum* cv. Raki, *Cynodon dactylon* var. Shangri-La, and *Zoysia japonica* "Sunrise Brand Zoysiagrass") were subjected to one night of cold (5±3ºC) and the photosynthetic rates and electron transport were studied on the subsequent first hours of the light period at the growth temperature (25ºC) under low, moderate and high light intensities (200, 530 and 1300 μmol m^{-2} s^{-1}, respectively). This study will allow to understand if the dark-chilling alters the sensitivity of the plants to an increase in the light intensity, as occurs in C_3 plants. It also allows to choose the light intensity at which the future metabolic and enzymatic studies will be performed in order to analyse the dark-chilling effects *per se*, avoiding the potential synergistic effects of dark-chilling and light intensity. Furthermore, this study may show the involvement of the electron

transport reactions and stomatal opening in the limitation of photosynthetic rate after dark-chilling.

3.3. Material and Methods

3.3.1. Plant material, growth conditions and dark-chilling stress induction

The plant material and growth conditions used here were described earlier in Chapter 2, as well as the dark-chilling stress imposition. The only difference being the number of chilling nights, since in this Chapter the plants were only subjected to one night of cold (5±3ºC). After night-chilling the plants were returned to the growth chamber, at a photosynthetic photon flux density (PPFD) of 250-300 μmol m^{-2} s^{-1} and 25ºC, and the gas-exchange and fluorescence measurements were performed from one to five hours after the beginning of the light period. As in Chapter 2, it was observed that the values obtained did not change during the first hours of the light period. The widest part of fully expanded leaves was used in all the assays.

3.3.2. Gas-exchange measurements

Gas-exchange measurements were performed using an LCpro+ portable infra red gas analyser (ADC BioScientific Ltd., Hoddesdon, Hertfordshire, UK) fitted with a broad leaf Parkinson chamber. Two sets of experiments were done, namely a gas-exchange light-response curve and a simultaneous determination of gas-exchange and chlorophyll *a* fluorescence parameters at three chosen light intensities. Steady-state measurements of net CO_2 assimilation rate (*A*) and stomatal conductance to water vapour (*gs*) were obtained at a CO_2 concentration of 350 μL L^{-1}, a leaf temperature of 25ºC and a relative humidity of 40 to 50%. In both sets of gas-exchange assays the measurements were performed after leaving the leaf 15 min at each light intensity, in order to attain steady-state values of photosynthesis. Furthermore, in both sets of experiments the gas-exchange measurements were followed by the leaf area determination (portable leaf area meter CI-202, CID Inc., Camas, Washington, USA).

The gas-exchange light-response curves were obtained via step-wise increases in irradiance from darkness to 1705 μmol m^{-2} s^{-1} using the infra red gas analyser external led light unit (ADC BioScientific Ltd., Hoddesdon, Hertfordshire, UK). The analysis of the photosynthetic light-response curves was performed through the program Statistics (version 8.0, 2007, StatSoft Inc., Tulso, Oklahoma, USA) and allowed the prediction of the apparent quantum yield (ϕ), curvature degree (θ), maximal rate of photosynthesis (*Amax*) and mitochondrial respiration (*Rd*) accordingly to von Caemmerer (2000) and Lambers *et al.* (1998) (Table 3.1.).

Table 3.1. Equation used to predict the maximal rate of photosynthesis (*Amax*), apparent quantum yield (ϕ), curvature degree (θ) and mitochondrial respiration (*Rd*) from the light-response curves of photosynthesis accordingly to von Caemmerer (2000) and Lambers *et al.* (1998). Abbreviations used: *A*, net CO_2 assimilation rate; *I*, irradiance used.

Parameters	Equation
Amax, ϕ, θ, Rd	$A = \frac{I \times \phi + A\max - \sqrt{(I \times \phi + A\max)^2 - 4 \times \theta \times I \times \phi \times A\max}}{2 \times \theta} - Rd$

The simultaneous gas-exchange and chlorophyll *a* fluorescence measurements were performed at three increasing light intensities (200, 530 and 1300 μmol m^{-2} s^{-1}) given by an external cold light source (KL 2500 LCD, Fiber Optics Schott AG, Mainz Germany). The three light intensities were chosen from the photosynthetic light-response curves, and correspond, respectively, to a light intensity in the initial slope, near the curvature zone and after the curvature zone of the photosynthesis curve.

Each value presented for each set of experiments corresponds to the mean of ten plants *per* treatment.

3.3.3. Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence measurements were performed simultaneously with the second set of gas-exchange experiments at the same three light intensities (200, 530 and 1300 μmol m^{-2} s^{-1}). The fluorescence optical fiber was oriented to the leaf with a 45º angle. As described for the second set of gas-exchange measurements, the actinic light

was provided through an external cold light source combined with a special fiber optics (460F, Heinz Walz GmbH, Effeltrich, Germany).

Photochemical parameters were measured using a pulse amplitude modulation fluorometer (PAM-101, Heinz Walz GmbH, Effeltrich, Germany) supplied with a PAM Data Acquisition System (PDA-100, Heinz Walz GmbH, Effeltrich, Germany). The fluorometer was operated by WinControl (V1.48 software, 2000, Heinz Walz GmbH, Effeltrich, Germany). Leaves were first dark-adapted for 10 min. The minimum fluorescence level was determined with a measuring light intensity lower than 1 μmol m^{-2} s^{-1}. The maximum fluorescence level was measured on dark-adapted leaves through a saturating light pulse of 4500 μmol m^{-2} s^{-1} for 1 s. The leaf was then continuously illuminated for 15 min at 200 μmol m^{-2} s^{-1}. The steady-state value of fluorescence was recorded and a second saturating pulse was imposed to determine the maximum fluorescence level in the first light-adapted state. Fluorescence levels returned to minimum values after 3 s illumination with far-red light. The steady -state fluorescence parameters at 530 and 1300 μmol m^{-2} s^{-1} were obtained by repeating the above procedure. The maximum photochemical efficiency of PSII reaction centres of dark-adapted leaves (*Fv/Fm*) was calculated according to Kitajima and Butler (1975). For each actinic light intensity the effective quantum yield of PSII electron transport (Φ_{PSII}) (Genty *et al.* 1989) and the photochemical and non-photochemical quenching coefficients, respectively *qP* and *NPQ* (Schreiber *et al.* 1986), were calculated. In order to understand if the electron transport was limiting the photosynthetic rate, the *ETR/A* ratio was calculated dividing the electron transport rate (*ETR*) values by four due to the need of four electrons to photosynthetically reduce one molecule of CO_2 (Hall and Rao 1999).

As in the gas-exchange measurements, each value presented corresponds to the mean of ten plants for each treatment.

3.3.4. Statistical analysis

The data were statistically analysed using parametric tests at a stringency of $P<0.05$ with the program Statistical Package for Social Sciences (SPSS) (version 15.0, 2006, SPSS Inc., Chicago, Illinois, USA). The variance in the mean data of photosynthetic light-response curve parameters and *Fv/Fm* was evaluated using a T-test. The Pearson

correlation coefficient was used to analyse the correlation between *A* and *gs* in the light-response curve assay. The simultaneous gas-exchange and fluorescence parameters (except the *Fv/Fm* analysis) mean values of control and one night-chilled plants at three light intensities were analysed through an ANOVA test followed by a Tukey HSD test, as well as the photosynthetic rate values at 200, 530 and 1300 μmol m^{-2} s^{-1} obtained from the light-response curves. All the analyses were performed for each species separately, what was evidenced by the use of small letters and apostrophes in the figures and tables.

3.4. Results

3.4.1. Light-response curves

Paspalum dilatatum, *C. dactylon* and *Z. japonica* presented distinct photosynthetic light-response curves (Fig. 3.1.*a*,*b*,*c*), both in control and one night-chilled plants.

The light-response curve plateau is reached at a lower light intensity in *Z. japonica* (Fig. 3.1.*a*,*b*,*c*). The analysis of the curve at three chosen light intensities, in the initial slope, curvature zone and after the curvature zone (respectively 200, 530 and 1300 μmol m^{-2} s^{-1}) (Table 3.2.), showed that the photosynthesis in *Z. japonica* plants is already saturated at 530 μmol m^{-2} s^{-1} both in control and chilled plants, but not in *P. dilatatum* and *C. dactylon*. Furthermore, *C. dactylon* was the species that presented higher rates of photosynthesis at 1300 μmol m^{-2} s^{-1} (Fig. 3.1.*a*,*b*,*c* and Table 3.2.).

One night chilling altered the response of photosynthesis to increasing light intensity in the three species under study (Fig. 3.1.*a*,*b*,*c* and Table 3.2.) only for light intensities higher than 200 μmol m^{-2} s^{-1}. Moderate light intensities (530 μmol m^{-2} s^{-1}) did not affect *A* after the dark-chilling only in *P. dilatatum* and *C. dactylon* (Table 3.2.). However, high light intensities (1300 μmol m^{-2} s^{-1}) decreased *A* in the three species after stress (Table 3.2.).

The light response-curve parameter analysis (Table 3.3.) show that ϕ and θ of control plants are similar in the three species studied, but *Amax* and *Rd* are different. The *Amax* and the *Rd* in the control plants were higher in *C. dactylon* and lower in *Z. japonica*. The parameters ϕ, θ and *Rd* were not affected by the short-term dark-chilling

stress. On the contrary, there was a decrease in the *Amax* of about 14%, 16% and 37%, respectively in *P. dilatatum*, *C. dactylon* and *Z. japonica* after one night-chilling (Table 3.3.).

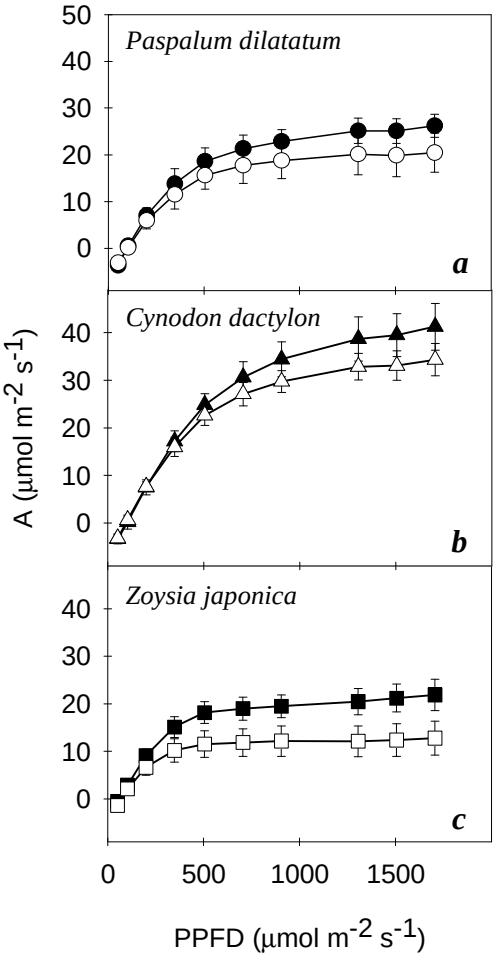

Figure 3.1. Light-response curve of net CO_2 assimilation rate (*A*) for control (closed symbols) and one night-chilled (open symbols) plants of *Paspalum dilatatum* (***a***), *Cynodon dactylon* (***b***) and *Zoysia japonica* (***c***). Measurements were performed under a CO_2 concentration of 350 µL L^{-1}, at 25ºC and at a PPFD from 0 to 1705 µmol m^{-2} s^{-1}. Data are mean ± SD of ten plants of each species *per* treatment. PPFD, photosynthetic photon flux density.

A positively and closely correlation was found between *A* and *gs*, both in control and chilled plants (Figure 3.2.). This result was expected since *gs* also increased curvilinearly with light intensity, presenting a similar chilling response to that of *A* (results not shown).

Three light intensities (200, 530 and 1300 μmol m^{-2} s^{-1}) were chosen from the light-response curves of photosynthesis (Fig. 3.1.) to perform the simultaneous gas-exchange (Fig. 3.3.) and chlorophyll *a* fluorescence (Fig. 3.4., Fig. 3.5.) measurements, as referred in the Materials and Methods section of this Chapter.

Table 3.2. The CO_2 assimilation rate values obtained at three light intensities (200, 530 and 1300 μmol m^{-2} s^{-1}) from the light-response curves (See Fig. 3.1.) for control and one night-chilled plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. Data are mean ± SD of ten plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at P<0.05.

	CO_2 assimilation rate (μmol m^{-2} s^{-1})		
	200 (μmol m^{-2} s^{-1})	530 (μmol m^{-2} s^{-1})	1300 (μmol m^{-2} s^{-1})
P. dilatatum			
Control	7.0 ± 1.63 a	18.6 ± 2.88 bc	25.2 ± 2.70 d
1 Night Chilling	5.9 ± 1.80 a	15.6 ± 1.92 b	20.1 ± 4.41 c
C. dactylon			
Control	7.5 ± 1.57 a’	24.8 ± 2.31 b’	38.7 ± 4.62 c’
1 Night Chilling	7.6 ± 0.99 a’	22.6 ± 2.09 b’	32.8 ± 2.78 d’
Z. japonica			
Control	9.1 ± 1.36 ab’’	18.2 ± 2.30 c’’	20.5 ± 2.80 c’’
1 Night Chilling	6.6 ± 1.63 a’’	11.5 ± 2.77 b’’	12.2 ± 3.23 b’’

3.4.2. Gas-exchange measurements at three light intensities

Simultaneous gas-exchange and fluorescence results show that *A* (Fig. 3.3.*a*,*b*,*c*) and *gs* (Fig. 3.3.*d*,*e*,*f*) increased with the three light intensities used, 200, 530 and 1300 μmol m^{-2} s^{-1} both in control and stressed plants. The three plants showed similar *A* values at low (200 μmol m^{-2} s^{-1}) and moderate (530 μmol m^{-2} s^{-1}) light intensities, however *C. dactylon* presented higher *A* values at the higher light intensity (1300 μmol m^{-2} s^{-1}).

The increase of *A* with light was higher in control plants than in one night-chilled plants of *P. dilatatum* and *Z. japonica*. In the three species studied, *A* was not altered by

dark-chilling at the lower light intensity. In *P. dilatatum* under this light condition there was a 30% decrease of *gs*. At the moderate and high light intensities a similar decrease of *A* and *gs* was found with chilling in *P. dilatatum* (25% and 20%, respectively for each light level) and *Z. japonica* (29% and 35%, respectively for moderate and high light intensities). In *C. dactylon* the short-term dark-chilling stress did not affect *A* or *gs* at any of the light intensities used.

Table 3.3. Maximal rate of photosynthesis (*Amax*), apparent quantum yield (ϕ), curvature degree (θ) and mitochondrial respiration (*Rd*) predicted from the photosynthetic light-response curve for control and one night-chilled plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica* (See Fig. 3.1.). Data are mean ± SD of ten plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at P<0.05.

	Amax (μmol m^{-2} s^{-1})	ϕ ($*10^2$ μmol μmol^{-1})	θ (relative units)	Rd (μmol m^{-2} s^{-1})
P. dilatatum				
Control	36 ± 3.1 a	8.7 ± 1.34 a	0.78 ± 0.110 a	7.8 ± 1.49 a
1 Night Chilling	31 ± 4.8 b	8.5 ± 1.67 a	0.81 ± 0.086 a	7.4 ± 1.87 a
C. dactylon				
Control	55 ± 6.5 a'	8.8 ± 1.17 a'	0.76 ± 0.103 a'	8.2 ± 1.15 a'
1 Night Chilling	46 ± 5.0 b'	8.7 ± 1.27 a'	0.75 ± 0.106 a'	7.8 ± 1.23 a'
Z. japonica				
Control	27 ± 3.8 a''	8.6 ± 1.91 a''	0.79 ± 0.130 a''	4.5 ± 0.97 a''
1 Night Chilling	17 ± 4.5 b''	6.4 ± 1.76 a''	0.91 ± 0.062 a''	4.5 ± 1.12 a''

3.4.3. Chlorophyll *a* fluorescence at three light intensities

In all three species a similar value for *Fv/Fm* (close to 0.8) was observed in the control leaves. The *Fv/Fm* decreased slightly (3%) in *P. dilatatum* under chilling conditions, but did not change with stress in *C. dactylon* and *Z. japonica* (Fig. 3.4.).

The ratio *ETR/A* decreased with the increase in light intensity in the three species (Table 3.4.). However, results showed that the electron transport rate is always higher than photosynthesis at any light intensity, except in *P. dilatatum* chilled plants at the higher illumination (Table 3.4.).

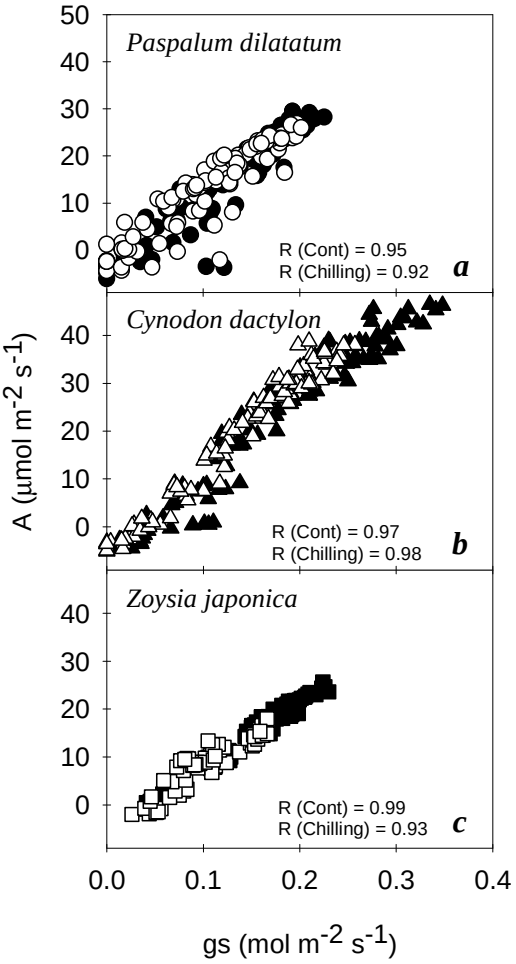

Figure 3.2. Relationship between net CO_2 assimilation rate (*A*) and stomatal conductance to water vapour (*gs*) and Pearson correlation coefficient (R) for control (closed symbols) and one night-chilled (open symbols) plants of *Paspalum dilatatum* (***a***), *Cynodon dactylon* (***b***) and *Zoysia japonica* (***c***). The Pearson correlation coefficient shows how related are both parameters. Data were taken from the light-response curve values obtained for the net CO_2 assimilation rate (See Fig. 3.1.) and stomatal conductance to water vapour (results not shown).

In all species, the Φ_{PSII} (Fig. 3.5.*a*,*b*,*c*) and the *qP* (Fig. 3.5.*d*,*e*,*f*) values of control leaves decreased as the light intensity increased, being this decrease higher at 1300 μmol m^{-2} s^{-1}. On the contrary, the *NPQ* (Fig. 3.5.*g*,*h*,*i*) values of control leaves increase with light intensity and this increase was higher at 1300 μmol m^{-2} s^{-1}.

Although Φ_{PSII}, *qP* and *NPQ* control values were similar in the three gramineae at the lower and moderate light intensities, Φ_{PSII} was much higher and *NPQ* was much lower in *C. dactylon* at the higher light intensity. While in *P. dilatatum* Φ_{PSII} decreased in chilled plants at any of the light intensities used (4%, 12% and 41% respectively at the low, moderate and high light intensities), in *C. dactylon* this parameter was not

affected by dark-chilling. In *Z. japonica* there was a decrease of Φ_{PSII} with stress (23%) only at the higher light intensity. Under chilling conditions, *qP* did not show a significant variation from the control (P>0.05) at any of studied light intensities. Dark-chilling did not affect *NPQ* in *P. dilatatum* and *C. dactylon* at the lower light intensity, but increased this parameter at moderate and high light intensities. *Zoysia japonica* did not show any change in *NPQ* with the imposed stress.

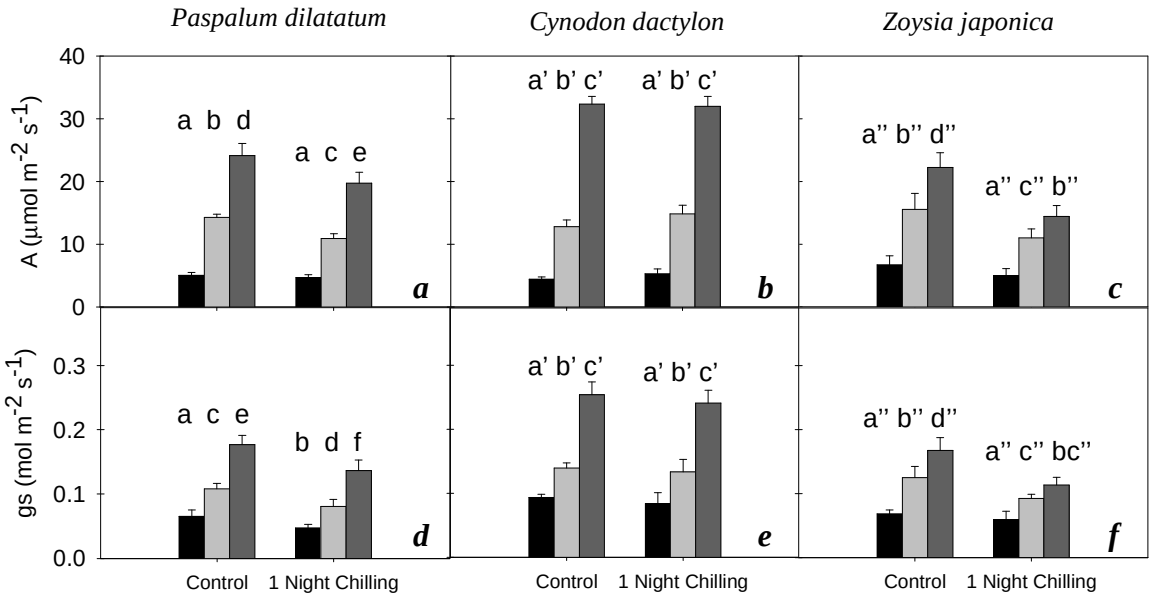

Figure 3.3. Net CO_2 assimilation rate (*A*; ***a***, ***b***, ***c***) and stomatal conductance to water vapour (*gs*; ***d***, ***e***, ***f***) at three light intensities for control and one night-chilled plants of *Paspalum dilatatum* (***a***, ***d***), *Cynodon dactylon* (***b***, ***e***) and *Zoysia japonica* (***c***, ***f***). Measurements were performed simultaneously with chlorophyll *a* fluorescence assays (See Fig. 3.4. and Fig. 3.5.) under a CO_2 concentration of 350 μL L^{-1}, at 25ºC and at a PPFD of 200 μmol m^{-2} s^{-1} (black bars), 530 μmol m^{-2} s^{-1} (grey bars) and 1300 μmol m^{-2} s^{-1} (dark grey bars). Data are mean ± SD of ten plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at P<0.05.

3.5. Discussion

3.5.1. Dark-chilling for one night did not decrease photosynthesis at low light intensity, but decreased at higher light intensities

Some differences were observed between the photosynthetic rates obtained from the light-response curves (Fig. 3.1. and Table 3.2.) and from the gas-exchanges measured simultaneously with fluorescence (Fig. 3.3.). These differences may be related

to the way light intensity was increased. Contrary to the sudden increase in light intensity in the simultaneous gas-exchange measurements (Fig. 3.3.), the increase in light intensity was gradual in the light-response curves (Fig. 3.1. and Table 3.2.), which may have allowed the plants to slowly adapt their photosynthetic metabolism in response to the increase in light. Although the small differences, both type of assays show that *A* is decreased in the three C_4 plants studied after one night-chilling in a manner that is dependent on the light intensity at which plants were subjected, as also observed by Feng and Cao (2005) in a C_3 species.

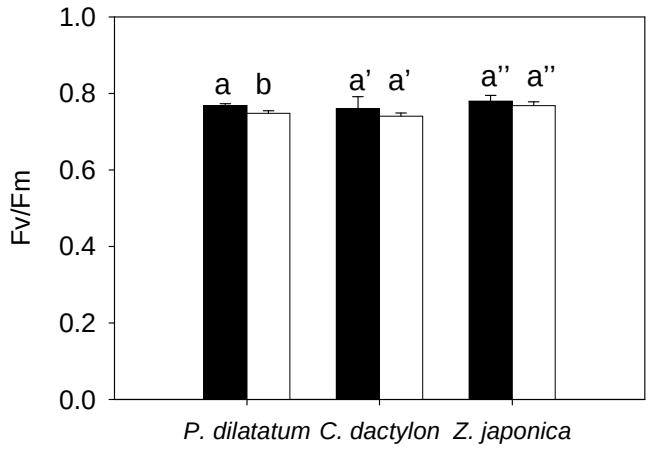

Figure 3.4. Maximum photochemical efficiency of PSII reaction centres of dark adapted leaves (*Fv/Fm*) of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica* control (black bars) and one night-chilled (white bars) plants. Measurements were performed simultaneously with gas-exchange measurements (See Fig. 3.3.). Data are mean ± SD of ten plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at P<0.05.

However, the velocity at which light intensity increased affected differently the photosynthetic rate only in *P. dilatatum* and in *C. dactylon* dark-chilled plants (Fig. 3.1., Fig. 3.3. and Table 3.2.). Furthermore, the differences observed between the two gas-exchange assays in both plants were obtained at different light intensities. Under stress *A* was not affected at 530 μmol m^{-2} s^{-1} and decreased at 1300 μmol m^{-2} s^{-1} in *P. dilatatum* when light was slowly raised (Fig. 3.1. and Table 3.2.). However, when light was suddenly increased, *A* was reduced at both light intensities in this species (Fig. 3.3.). Contrary to *P. dilatatum*, where the differences between the two assays were observed at 530 μmol m^{-2} s^{-1}, in *C. dactylon* they were found at 1300 μmol m^{-2} s^{-1}, with

a decrease in *A* observed only when the raise in light was slowly imposed (Fig. 3.1., Fig 3.3. and Table 3.2.). These results suggest that each species present a different sensitivity to the way light is increased.

Table 3.4. Ratio between electron transport rate (*ETR*, results not shown) and net CO_2 assimilation rate (*A*, See Fig. 3.5.) at three different light intensities (200, 530 and 1300 μmol m^{-2} s^{-1}) for control and one night-chilled plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. The *ETR/A* ratio was calculated dividing the electron transport rate by four due to the need of four electrons to photosynthetically reduce one molecule of CO_2 (see this Chapter Material and Methods). Data are mean ± SD of ten plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at P<0.05.

	ETR/A		
	P. dilatatum	*C. dactylon*	*Z. japonica*
Control			
200 μmol m^{-2} s^{-1}	3.0 ± 0.37 a	3.3 ± 0.37 a’	2.3 ± 0.18 a’’
530 μmol m^{-2} s^{-1}	2.4 ± 0.24 b	2.4 ± 0.32 b’	2.2 ± 0.19 a’’
1300 μmol m^{-2} s^{-1}	1.3 ± 0.08 c	1.6 ± 0.10 c’	1.3 ± 0.11 b’’
1 Night Chilling			
200 μmol m^{-2} s^{-1}	3.2 ± 0.41 a	2.9 ± 0.41 a’	2.4 ± 0.31 a’’
530 μmol m^{-2} s^{-1}	2.6 ± 0.23 b	2.3 ± 0.22 b’	2.1 ± 0.14 a’’
1300 μmol m^{-2} s^{-1}	0.9 ± 0.10 d	1.4 ± 0.13 c’	1.4 ± 0.16 b’’

In *Z. japonica*, the different way light intensity was increased just altered the irradiance at witch photosynthesis saturated, at 530 μmol m^{-2} s^{-1} when the increase light was imposed gradually (Fig. 3.1. and Table 3.2.) and at higher values when it was imposed suddenly (Fig. 3.3.). In this species, dark-chilling decreased *A* in both experiments at 530 and 1300μmol m^{-2} s^{-1}.

Photosynthesis on the following warm light period was not affected by dark-chilling when measured at low light intensity in the three species under study (Fig. 3.3. and Table 3.2.), a result that was supported by the non-variation of ϕ values between control and chilled plants (Table 3.3.). However, the decrease of *A* in *P. dilatatum* and *Z. japonica* at moderate and/or high light intensities (Fig. 3.3. and Table 3.2.) and the decrease in *Amax* in the three species (Table 3.3.) after one night-chilling indicates that

stress altered the capacity of these C_4 plants to face an increase in light. This alteration may have occurred by an enhancement of the susceptibility of plants to photoinhibition and/or by a decrease in the photosynthetic capacity at enzyme level. Several authors have also reported in C_3 and C_4 species decreases in the photosynthetic rate at moderate and high light intensities after a dark-chilling stress of one night (e.g. Ivory and Whiteman 1978, Bauer *et al.* 1985, Pittermann and Sage 2001, van Heerden *et al.* 2003*a*) and several nights (e.g. Flexas *et al.* 1999, van Heerden and Krüger 2002, van Heerden *et al.* 2003*a*).

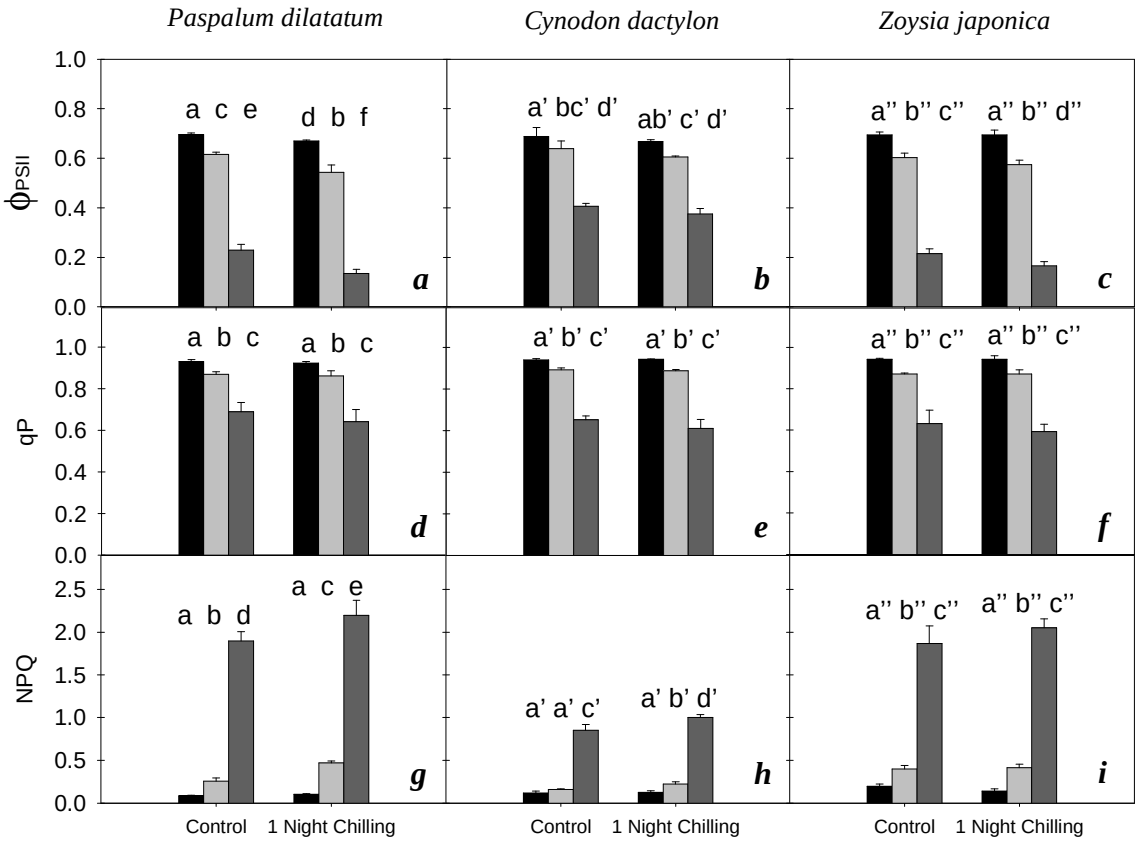

Figure 3.5. Effective quantum yield of PSII electron transport (Φ_{PSII}; ***a***, ***b***, ***c***), photochemical quenching coefficient (*qP*; ***d***, ***e***, ***f***) and non-photochemical quenching coefficient (*NPQ*; ***g***, ***h***, ***i***) at three light intensities for control and one night-chilled plants of *Paspalum dilatatum* (***a***, ***d***, ***g***), *Cynodon dactylon* (***b***, ***e***, ***h***) and *Zoysia japonica* (***c***, ***f***, ***i***). Measurements were performed simultaneously with gas-exchange measurements (See Fig. 3.3.) at an actinic light intensity of 200 μmol m^{-2} s^{-1} (black bars), 530 μmol m^{-2} s^{-1} (grey bars) and 1300 μmol m^{-2} s^{-1} (dark grey bars). Data are mean ± SD of ten plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at $P<0.05$.

The three species studied showed a different sensitivity of the photosynthetic process to light intensity after the dark-chilling. The higher vulnerability of this process was found in *Z. japonica*, followed by *P. dilatatum*, while *C. dactylon* presented the more stable photosynthetic metabolism after dark-chilling. In a previous study we have also found that *C. dactylon* photosynthesis was less sensitive to a drought stress than *P. dilatatum* and *Z. japonica* (Carmo-Silva, Soares *et al.* 2007). The higher sensitivity of *Z. japonica* plants to dark-chilling may be related with the cold sensitivity of the enzyme PEPCK to chilling conditions (Matsuba *et al.* 1997).

Low temperature is known to decrease respiration (Atkin and Tjoelker 2003). For example, both a decrease in cytochrome *c* oxidase and ATPase activities have been observed in maize seedlings after a short-term chilling stress (Prasad *et al.* 1994). However, this process may be quickly and fully reversed after transference to warm temperatures for one hour, as found in cold-sensitive mung bean dark-chilled plants (Yoshida *et al.* 1989, Yoshida 1991). In accordance, dark-chilling did not affect mitochondrial respiration measured after one to five hours of exposure to warm temperatures at low light intensity (Fig. 3.1. and Table 3.3.).

3.5.2. High correlation between the decrease in photosynthetic rate and stomatal closure after dark-chilling

The high correlation between *A* and *gs* in control and chilled plants at different light intensities (Fig. 3.2., Fig. 3.3.) indicates that stomatal limitations to photosynthesis may have occurred upon rewarming *P. dilatatum* and *Z. japonica* plants, as observed in several dark-chilled C_4 and C_3 plants (e.g. Ivory and Whiteman 1978, Martin *et al.* 1981, Bauer *et al.* 1985, van Heerden *et al.* 2003*a*, Feng and Cao 2005). In *C. dactylon*, dark-chilling stress did not affect *A* or *gs* up to a light intensity of 1300 μmol m^{-2} s^{-1} (Fig. 3.3.), as also observed in the less sensitive soybean genotype Mapple Arrow (van Heerden *et al.* 2003*a*). The decrease of *gs* without affecting *A* at low light intensity on dark-chilled *P. dilatatum* plants (Fig. 3.3.) may occur in C_4 species due to the presence of a CO_2 concentration mechanism that buffers small changes in CO_2 concentration. Furthermore, this result shows that in *P. dilatatum* stomata were firstly affected by the dark-chilling stress. Stomatal closure is not surprising to occur when the whole plant is subjected to low night temperatures since chilling is known to reduce the hydraulic

conductivity of roots (Rab and Saltveith 1996 and references therein), decreasing water uptake.

3.5.3. Dark-chilling induced alterations in PSII activity, less evident at low light intensity

Cynodon dactylon and *Z. japonica* one night-chilled plants did not show photoinhibition of the photosynthetic process (measured by *Fv/Fm* ratio) (Fig. 3.4.), indicating no damage at the level of D1 protein at the growth low light intensity (250-300 μmol m^{-2} s^{-1}). Although no photoinhibition of the photosynthetic process has been found in *P. dilatatum*, small changes may have occurred in dark-chilled plants of this species at the D1 level. A decrease in *Fv/Fm* and in the content of D1 protein was found in C_3 dark-chilled plants when illuminated at high light intensity (Bertamini *et al.* 2006).

Dark-chilling enhanced the susceptibility of the electron transport rate to the increased in light intensity on the subsequent warm day (results not shown) in *P. dilatatum* and *Z. japonica*, as also shown by Feng and Cao (2005) in C_3 plants. However, the electron transport only limited the photosynthetic rate in dark-chilled *P. dilatatum* at the higher light intensity (Table 3.4.). In dark-chilled *Z. japonica* plants the electron transport was not limiting the CO_2 assimilation rate, as also shown by Kee *et al.* (1986) and van Heerden *et al.* (2004) in C_3 plants.

Several fluorescence studies performed at moderate and high light intensities showed that dark-chilling greatly damage the electron transport chain at the PSII level (e.g. van Heerden *et al.* 2003*a*, van Heerden *et al.* 2003*b*, van Heerden *et al.* 2004, Garstka *et al.* 2007). The non variation of Φ_{PSII} with the stress at any light intensity in *C. dactylon* shows that this species presents the least sensitive PSII activity after a dark-chilling. On the contrary, *P. dilatatum* dark-chilled plants presented the most sensitive PSII activity (Fig. 3.5.). The non variation of *qP* with the stress in the three species, suggest that the decrease in PSII efficiency observed at any light intensity for *P. dilatatum* or at high light intensity for *Z. japonica* night-chilled plants occurred at the primary charge separation level. Dark-chilling and subsequent warming at high light intensity has been shown to uncouple the oxygen-evolving complex (Shen *et al.* 1990, Strauss *et al.* 2007), decreasing Hill activity in cold-sensible species (e.g. Kaniuga *et al.* 1978, Bertamini *et al.* 2005, Bertamini *et al.* 2006), but generally leaving other parts of

the electron transport unaffected (Kee *et al.* 1986, Bertamini *et al.* 2005, Bertamini *et al.* 2006).

Damage at the primary charge separation level suggests that dissipation of excess energy needed to occur at the PSII antenna level. In accordance with Flexas *et al.* (1999) and Feng and Cao (2005), it was observed an increase in *NPQ* in *P. dilatatum* dark-chilled plants at moderate and high light intensities and a tendency to increase in *Z. japonica* at the higher light intensity (Fig. 3.5.).

3.6. Conclusion

The decrease of photosynthesis after one night-chilling, involving at least stomatal limitations, depends on the light intensity on the subsequent warm light period. Low light intensity did not affect the photosynthetic rate after one night-chilling in the three C_4 species studied. Although alterations in PSII activity of dark-chilled plants were mostly observed at moderate and high light intensities in *P. dilatatum* and *Z. japonica*, the former species showed some PSII damaged even at low light intensity. Moderate and high light intensities decreased photosynthetic rate in *P. dilatatum* and *Z. japonica*, while in *C. dactylon* higher light intensities were needed. Electron transport rate may limit photosynthetic rate at high light intensity only in dark-chilled *P. dilatatum* plants. Results suggest that one night-chilling turned the overall photosynthetic process more susceptible to light, indicating that some metabolic alterations must have occurred in response to the imposed stress. Since the authors aimed to understand the dark-chilling effects *per se*, i.e. avoiding the potential synergistic effects of dark-chilling and light intensity, it was chosen to perform the future metabolic and enzymatic studies at a low light intensity, similar to that of growth conditions.

3.7. References

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Chapter 4.
One night-chilling effects on leaf carbohydrates, fatty acid composition and carboxylating enzymes in C_4 gramineae from different metabolic sub-types.

The results presented in this Chapter were obtained by Ana Sofia Soares at the laboratory of the Secção de Fisiologia e Bioquímica Vegetais, Departmento de Biologia Vegetal, Faculdade de Ciências, Universidade de Lisboa.

A manuscript to submit for publication in an international peer-reviewed journal is being prepared with part of the results presented in this Chapter, together with part of those presented in Chapter 2 and Chapter 3. These results were also presented in the following conference:

- Soares AS, Bernardes da Silva A, Marques da Silva J, Arrabaça, MC (2006) Down-regulation of photosynthesis in C_4 species from different metabolic subtypes to dark-chilling. In: RAS03 – Ecophysiology, Whole-Plant Approaches, Abstract book of the XV Congress of the Federation of European Societies of Plant Biology (FESPB), Lyon, França – Panel Presentation.

4. One night-chilling effects on leaf carbohydrates, fatty acid composition and carboxylating enzymes in C_4 gramineae from different metabolic sub-types.

4.1. Abstract

In this Chapter the effects of one night-chilling was studied on the leaf carbohydrates and protein content, membrane parameters and fatty acid composition, and on the activity of phospho*enol*pyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The studies were performed on the following warm (25ºC) light period after one night-chilling (5±3ºC) in C_4 grasses of the three metabolic sub-types, *Paspalum dilatatum* Poiret (NADP-ME), *Cynodon dactylon* (L.) Pers (NAD-ME) and *Zoysia japonica* Steudel (PEPCK). The dark-chilling led to an increase in the content of soluble and insoluble carbohydrates in all species studied, but this increase was higher in *P. dilatatum* plants. The total soluble protein content was not affected by the chilling stress, while the total fatty acid content increased significantly in *C. dactylon* and *Z. japonica* and tended to increase in *P. dilatatum*. The total fatty acid unsaturation degree, which was always higher in *Z. japonica* and lower in *C. dactylon*, tended to increase in response to the dark-chilling stress. Membrane integrity increased after the short-term dark-chilling in all species, which may be related to the increase in lipid content. In contrast to the other species, in *Z. japonica* it was found a decrease in lipid peroxidation after dark-chilling. Carboxylating enzyme activities were differently affected by one night-chilling among the C_4 species studied, but generally no decrease was observed. Results show that one night of chilling altered the metabolism of the C_4 plants studied on the subsequent warm light period.

Keywords: carbohydrates content, C_4 plants, electrolyte leakage, lipid peroxidation, fatty acids, one night-chilling, PEPC, Rubisco.

4.2. Introduction

Chilling induce on plants structural, developmental, physiological, cellular and molecular alterations (Lyons 1973, Smallwood and Bowles 2002, Sung *et al.* 2003). All these responses depend on a common primary mechanism that involves a decrease in membrane function (Lyons 1973). Membranes are the first structure to be affected by low temperature (for review see Nishida and Murata 1996), and it has been shown that alterations in membrane fluidity, cytoskeleton rearrangement and calcium influx are the earliest events on the signal transduction pathway of cold stress (Smallwood and Bowles 2002, Bracale and Coraggio 2003). Low temperature induces a transition from a high fluid liquid crystalline phase to a more rigid gel phase, decreasing membrane fluidity (Murata and Los 1997). In order to maintain the membrane physical properties, one of the first responses of tolerant plants is an increase in lipids unsaturation (for review see Nishida and Murata 1996), namely an increase in linolenic acid.

However, as far as we know, there are no studies on fatty acid content and composition after a chilling treatment only during the night period. Under these conditions, an oxidative damage has been observed in soybean plants (van Heerden and Krüger 2002) and tropical C_3 trees (Jun *et al.* 2001). Reactive oxygen species (ROS) formed may react with fatty acids, increasing lipid peroxidation, as observed in dark-chilled C_3 trees (Jun *et al.* 2001) and in maize short-term chilled plants during the day and night periods (Chen *et al.* 2000).

It is also known that low temperature influences carbohydrate metabolism and protein synthesis, although few studies exist on the effect of dark-chilling on those processes. A rapid shift of plants to low temperature usually diminishes both sink processes and export of carbohydrates from leaves, disrupting the balance between supply and demand for assimilated carbon (Azcón-Bieto and Osmond 1983). Although Potvin *et al.* (1985) have observed that the translocation of photoassimilates decreased after dark-chilling in C_4 species, van Heerden *et al.* (2003*b*) showed no variation of starch and sucrose content on the following warm day period in two soybean plants after one to three nights of chilling temperatures. However, an accumulation of carbohydrates, namely sucrose, has been observed in response to low temperature in several C_3 chilling-tolerant species (Pollock 1986, Guy *et al.* 1992, Strand *et al.* 1999), but not in chilling-sensitive coffee plants (Ramalho *et al.* 2003). Furthermore, Taylor *et*

al. (1972) and Cavaco (2000) showed that *P. dilatatum* plants (C_4 NADP-malic enzyme, NADP-ME), respectively subjected to a short-term chilling or cold-acclimated, presented an accumulation of soluble and insoluble carbohydrates. Chilling injured plants show an inhibition of protein synthesis and an increased degradation of existing proteins (Potvin *et al.* 1984, Bredenkamp and Baker 1994). In accordance, low temperatures for three consecutive nights showed to decrease the soluble protein content in chilling-sensitive soybean plants (Strauss *et al.* 2007). In contrast, in cold-tolerant species (Naidu *et al.* 2003) the soluble protein content increased under cold acclimation due to the formation of several proteins (for review see Bracale and Coraggio 2003).

In addition, in C_3 plants dark-chilling is also known to alter the circadian regulation of sucrose phosphate synthase (EC 2.4.1.14) and nitrate reductase (EC 1.6.6.1) activities (Jones *et al.* 1998) and of photosynthetic gene transcription (Martino-Catt and Ort 1992). Furthermore, it has been reported to diminish the activity of fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11) and NADP-malate dehydrogenase (NADP-MDH, EC 1.1.1.82) (Jun *et al.* 2001, van Heerden *et al.* 2003*a*, van Heerden *et al.* 2003*b*). The activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) also decreased after two consecutive nights of chilling in cucumber plants (Zhou *et al.* 2004), but was not affected in soybean plants by one to three nights of chilling (van Heerden *et al.* 2003*b*). The only study on non-stomatal limitations to photosynthesis upon exposure to a dark-chilling stress in C_4 plants was performed by Pittermann and Sage (2001), showing that Rubisco activity decreased in the NAD-malic enzyme (NAD-ME) species *Muhlenbergia montana* (Nutt.). However, in C_4 plants other enzymes such as FBPase, Rubisco, phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31), phosphopyruvate dikinase (EC 2.7.9.1) and NADP-MDH may reduce their activity after a short-term chilling during one or more periods of 24 h (Taylor *et al.* 1974, Kingston-Smith *et al.* 1997) and during 4 h only in the light (Du *et al.* 1999).

Although the results from Chapter 3 indicate that the photosynthetic rate was not affected by dark-chilling at low growth light intensity, metabolic changes must have occurred that increased the susceptibility of plants to an increase in light intensity. The effects of one night-chilling (5±3ºC) on the carbohydrate and soluble protein content, electrolyte leakage, lipid peroxidation, total fatty acid content and composition, as well as PEPC and Rubisco activities, were studied in three C_4 warm-grasses, *Paspalum dilatatum* cv. Raki, *Cynodon dactylon* var. Shangri-La and *Zoysia japonica* "Jacklin

Sunrise Brand". Assays were performed at the low growth light intensity of 250-300 μmol m^{-2} s^{-1} in order to avoid the cumulative effects of chilling and high irradiance, studying only the dark-chilling effects *per se*.

4.3. Material and Methods

4.3.1. Plant material, growth conditions and dark-chilling stress imposition

The plant material and growth conditions, as well as the dark-chilling stress imposition of one night, were described earlier in Chapters 2 and 3. After one night-chilling (5±3ºC) the plants were returned to the growth chamber and samples for proteins, carbohydrates, electrolyte leakage, lipid peroxidation, fatty acid composition and enzyme activities were collected from one to two hours after the beginning of the light period at a light intensity of 250-300 μmol m^{-2} s^{-1}. As in Chapters 2 and 3, it was observed that the values obtained from the above assays did not change during the first hours of the light period. All the assays were performed on the middle and widest part of fully expanded leaves. Leaf samples for the *in vitro* assays were collected inside the environmental chamber, quickly frozen in liquid nitrogen and stored at -80ºC after determination of the fresh weight (*FW*) and the leaf area (portable leaf area meter CI-202, CID Inc., Camas, Washington, USA).

4.3.2. Determination of soluble and insoluble carbohydrates

Leaf soluble carbohydrates of control and one night-chilled plants were determined through an alcoholic extraction following the method described by Arrabaça (1981) with some modifications. The frozen samples were boiled for two min in 4 mL ethanol 80% (v/v) and the extract was collected to a graduated centrifuge tube. A further 2 mL ethanol 80% (v/v) was added to the leaf sample and boiled again for more two min. This extract was added to the first one. This procedure was repeated twice, being the final ethanol 80% (v/v) volume made up to 10 mL. Activated carbon was then added to clean the extract from leaf pigment colour. Afterwards, the extract was allowed to pass through a 0.45 μm diameter filter (Minisart RC25, Sartorius AG, Goettingen, Germany)

and was then dried at 80ºC under a nitrogen atmosphere. Deionised water (1 mL) was added to each tube to solubilize the carbohydrates. The leaf soluble carbohydrate content was analysed in Estação Agronómica Nacional (Oeiras, Portugal) by high performance liquid chromatography (Waters Corporation, Milford, Massachusetts, USA), equipped with a refraction index detector (model 2414, Waters Corporation, Milford, Massachusetts, USA). Separation was performed using a SugarPak 1 column (300 x 6.5 mm, Waters Corporation, Milford, Massachusetts, USA) at 90ºC. The elluent used was deionised water (containing 50 μL L^{-1} EDTA-Ca) at a flow rate of 0.5 mL min^{-1}. The identification and quantification of the sugars was carried out using known standards (Sigma, Sigma-Aldrich Co. Ltd., Gillingham, Dorset, UK).

The insoluble carbohydrate starch was extracted from the boiled leaf samples used above. After the alcoholic extraction, leaves were ground and let to dry in an oven at 60ºC for 24 h. The samples were then resuspended in 1mL deionised water and boiled for 30 min in order to gelatinize the starch. This was enzymatically digested overnight with 5U/mL amyloglucosidase (Sigma) and 5U/mL α-amilase (Sigma) in 25 mM acetate buffer (pH 4.5) at room temperature. After adding the activated carbon to the mixture, the extract was centrifuged for 5 min at 12000g (Sigma 2-16K, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at room temperature and the supernatant used for glucose determination by the dinitrosalicylic acid (DNS) assay (Chaplin 1986) adapted to a microplate reader (MPR-A4, DuPont, Belgium).

Each value is the mean of 14 plants *per* treatment.

4.3.3. Determination of soluble protein content

The total leaf soluble protein content of control and one night-chilled plants was determined by adding the samples to a cold mortar containing quartz sand, 1% (w/v) Polyclar AT and 20 mL/g *FW* of ice-cold extraction medium containing 50 mM HEPES-KOH pH 7.3, 1 mM EDTA, 5% (w/v) PVP25, 6% (w/v) PEG_{4000}, 10 mM DTT, 1% (v/v) protease cocktail inhibitor (Sigma) and 0.5% (v/v) Triton X-100. After grinding to obtain a fine suspension the homogenate was centrifuged for 1 min at 14000g at 4ºC (Sigma 2-16K, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), and the supernatant collected. Total leaf soluble protein was determined

according to the method of Bradford (1976) adapted to a microplate reader, using as standard bovine serum albumin (BSA).

Each value is the mean of ten plants *per* treatment.

4.3.4. Determination of fatty acids content

Frozen leaves of control and one night-chilled plants were boiled for 2 min in deionised water in order to stop lipolytic activities. Lipids were extracted in chloroform/methanol/water (1/1/1, v/v/v) accordingly to Bligh and Dyer (1959). Fatty acids were methylated with 5% sulphuric acid in methanol using the method of Metcalfe *et al.* (1966) after adding heptadecanoic acid (C17:0) as an internal standard. The fatty acids were quantified by gas liquid chromatography (Unicam 610 Series Gas Chromatograph, Unicam Ltd., UK) in Estação Agronómica Nacional (Oeiras, Portugal), as previously described (Campos *et al.* 2003) and were identified by comparison with a fatty acid methyl esthers standard (Sigma). The value for total fatty acids corresponds to the sum of the major individual fatty acids. The double bond index (DBI) was calculated as follows: DBI = (% monodienoic acids) + (2 * % dienoic acids) + (3 * % trienoic acids).

Each value is the mean of three plants for each treatment.

4.3.5. Electrolyte leakage

Each fresh leaf of control and one night-chilled plants was transversely cut into 0.5 cm pieces (total leaf length of 5 cm). Electrolyte leakage was measured as described by Campos *et al.* (2003) and Kocheva *et al.* (2004) with some modifications. The leaf pieces were rinsed three times in deionised water and subsequently floated on 3 mL of deionised water for 24 h at room temperature. After this time period (T0) the conductivity of the solution was measured using a condutivimeter (Con 5, Eutech Instruments Pte Ltd./Oakton Instruments, Singapure). Total conductivity was measured in the solution after heating the samples at 90ºC for 2 h (T1). Results were expressed as percentage of total conductivity as follows: % Conductivity = (Conductivity T0*100)/Conductivity T1.

Each value is the mean of 12 plants for each treatment.

4.3.6. Lipid peroxidation

For the measurement of lipid peroxidation the thiobarbituric acid reacting substances protocol (TBARS) was followed, accordingly to Hodges *et al.* (1999). This method allows the correction of the absorbance at 532 nm for other compounds (anthocyanins and carbohydrates) that interfere with measurements at this wavelength (Taulavuori *et al.* 2001). Frozen leaves of control and one night-chilled plants were homogenized in ethanol 80% (v/v) (20 mL/g *FW*) in a cold mortar containing quartz sand. The homogenate was centrifuged at 14000g for 5 min at 4ºC. A 1 mL aliquot of the supernatant was added to a test tube with 1 mL of either (a) "– TBA" solution (20% (w/v) trichloroacetic acid and 0.001% (w/v) butylated hydroxytoluene) or (b) "+ TBA" solution (containing the above compounds plus thiobarbituric acid (TBA) 0.65% (w/v)). Samples were then mixed and heated at 95ºC using a block heater (Tembloc, JP Selecta, S.A., Barcelona, Spain) for 25 min. The test tubes were cooled in ice to stop the reactions and were centrifuged at 14000g for 10 min at 4ºC. The absorbance of the supernatant was spectrophotometrically (Unicam UV/Vis 8755, Unicam Limited, Cambridge, UK) assayed at three distinct wavelengths: 440 nm, 532 nm and 600 nm. The malondialdehyde (MDA) equivalents present in the leaf samples tubes were calculated, after subtracting the absorbance at each wavelength of the correspondent background, in the following manner: MDA equivalents (nmol mL^{-1}) = [(A-B)*10^6]/157000, where A = [[(Abs_{532} +TBA) - (Abs_{600} +TBA)] – [(Abs_{532} –TBA) - (Abs_{600} –TBA)]] and B = [[(Abs_{440} +TBA) – (Abs_{600} +TBA)]*0.0571].

Each value is the mean of six plants for each treatment.

4.3.7. Enzyme extraction

Frozen samples of control and one night-chilled plants were ground to a fine suspension in a cold mortar containing quartz sand, 1% (w/v) Polyclar AT and ice-cold extraction medium (20 mL/g *FW*) containing 50 mM HEPES-KOH pH 7.3, 1 mM EDTA, 5% (w/v) PVP25, 6% (w/v) PEG_{4000}, 10 mM DTT, 1% (v/v) protease cocktail inhibitor (Sigma) and 0.5% (v/v) Triton X-100, as described by Carmo-Silva, Soares *et al.* (2007). The homogenate was centrifuged for 5 min at 14000g at 4ºC and the

supernatant was used immediately for measuring the activities of phospho*enol*pyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco).

4.3.8. Determination of PEPC activity

PEPC physiological and maximal activities (*Vphysiol* and *Vmax*, respectively) were measured spectrophotometrically (Unicam UV/Vis 500, Unicam Thermo Electron Corporation, Cambridge, UK) in a continuous assay at 340 nm and 25ºC according to Bakrim *et al.* (1992) with some modifications. For determination of *Vphysiol* the reaction mixture (1 mL) consisted of 50 mM HEPES-KOH pH 7.2, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 2.5 mM PEP (Sigma), 12 units of MDH (Sigma) and 20 µL of crude extract. The *Vmax* was measured in a similar reaction mixture, except that buffer pH was 8.0 and PEP concentration was 10 mM. In both cases, the reaction was started by the addition of 0.2 mM (final concentration) NADH (Sigma). The enzyme activation state was calculated as the ratio *Vphysiol*/*Vmax* *100.

Each value presented corresponds to the mean of eight plants for each treatment. Three replicate of the same extract were used.

4.3.9. Determination of Rubisco activity

Rubisco initial and total activities (*Vi* and *Vt*, respectively) were assayed at 25ºC by $^{14}CO_2$ incorporation into acid-stable products according to Parry *et al.* (1997) with some modifications. For both activities, the assay medium consisted of 50 mM HEPES-KOH pH 8.0, 30 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (0.5 µCi µmol^{-1}) (Amersham Biosciences, Piscataway, New Jersey, USA), and the reaction was stopped after 60 s through the addition of 100 µL of 2 N HCl. For *Vi* determination (final volume of 600 µL), the reaction was started by the addition of 50 µL of crude extract to the assay medium containing 0.5 mM RuBP (Sigma). For *Vt* determination (final volume of 600 µL) the crude extract (50 µL) was allowed to activate for 3 min in the assay medium, allowing the carbamylation of the available catalytic sites, after which the reaction was started by the addition of 0.5 mM RuBP (Sigma). Samples were completely dried at 60ºC and the residue resuspended in 1 mL of deionised water. The mixture was mixed with 8 mL of scintillation liquid (OptiPhase "HiSafe" 3, Perkin Elmer Life and Analytical Sciences,

Inc., Massachusetts, USA) and the radioactivity in the acid-stable products was measured by scintillation counting (LS 7800, Beckmann Instruments Inc., California, USA). The enzyme activation state was calculated as the ratio *Vi*/*Vt* *100.

Each value presented corresponds to the mean of eight plants for each treatment. Three replicate of the same extract were performed.

4.3.10. Statistical analysis

The data were statistically analysed using parametric tests at a stringency of $P<0.05$ with the program Statistical Package for Social Sciences (SPSS) 15.0, 2006 (SPSS Inc., Chicago, Illinois, USA). The significance of variation in the mean values for all the assays was evaluated through a T-test. All the analyses were performed for each species separately, what was evidenced by the use of small letters and apostrophes in the figures and tables.

4.4. Results

4.4.1. Leaf carbohydrate and soluble protein content

Paspalum dilatatum was the species that presented the lowest content of soluble (glucose, fructose and sucrose) carbohydrates in control plants. A similar content of these carbohydrates was found in *C. dactylon* and *Z. japonica* control plants (Fig. 4.1.), except that the former species presented a much higher content of glucose both in control and chilled plants (Fig. 4.1.*a*). The three species presented a similar content of insoluble carbohydrates (starch) in control plants. Both the leaf soluble and insoluble carbohydrates increased after one night-chilling in all species (Fig. 4.1.). The highest increase of soluble sugars after dark-chilling was found in *P. dilatatum* (10, 2 and 5 times, respectively for glucose, fructose and sucrose) (Fig. 4.1.*a*,*b*,*c*). Glucose and sucrose contents increased, respectively, by 40% and 100% both in *C. dactylon* and *Z. japonica* plants after one-night chilling (Fig. 4.1.*a*,*c*). On the contrary, fructose content increased more in *C. dactylon* than in *Z. japonica* (40% and 15%, respectively) (Fig.

4.1.*b*). Starch content increased 115%, 79% and 50% in one night-chilled plants of *P. dilatatum*, *C. dactylon* and *Z. japonica*, respectively (Fig. 4.1.*d*).

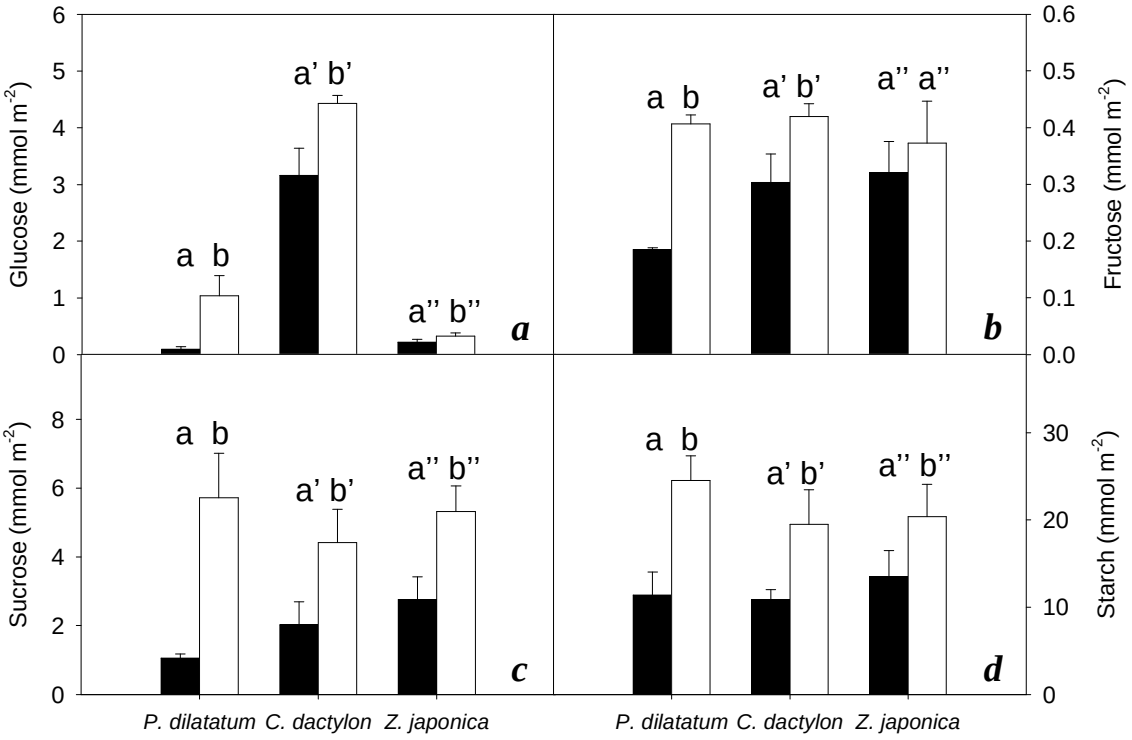

Figure 4.1. Leaf carbohydrates content of control (black bars) and one night-chilled (white bars) plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. Data for soluble (glucose, ***a***; fructose, ***b***; sucrose, ***c***) and insoluble (starch, ***d***) carbohydrates correspond to the mean ± SD of 14 plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at P<0.05.

The ratio of soluble to insoluble carbohydrates in control plants was about 0.12 ± 0.015, 0.50 ± 0.045 and 0.24 ± 0.027 (mean ± standard deviation), respectively in *P. dilatatum*, *C. dactylon* and *Z. japonica*. In one night-chilled plants there was a higher increase in the soluble than in the insoluble carbohydrates in *P. dilatatum* (soluble/insoluble ratio of 0.29 ± 0.030), followed by *Z. japonica* (soluble/insoluble ratio of 0.30 ± 0.034). On the contrary, in *C. dactylon* dark-chilled plants the increase in the soluble in relation to the insoluble carbohydrates was similar (soluble/insoluble ratio of 0.48 ± 0.049).

Total leaf soluble protein (Fig. 4.2.) was not affected by dark-chilling in any of the species studied and showed similar values among species.

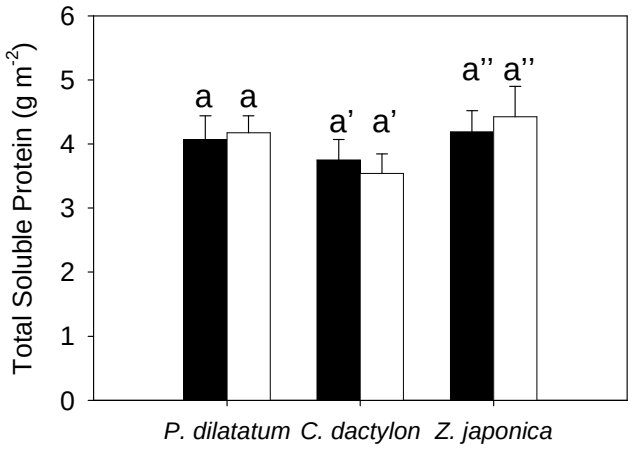

Figure 4.2. Total leaf soluble protein content of control (black bars) and one night-chilled (white bars) plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. Data are mean ± SD of ten plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at P<0.05.

4.4.2. Fatty acids content and composition

Under control conditions *C. dactylo*n presented the highest (17 mg m^{-2}) while *Z. japonica* the lowest (11 mg m^{-2}) total fatty acid content (Fig. 4.3.). After one night-chilling it was observed a significant total leaf fatty acid increase in *C. dactylon* (22%) and *Z. japonic*a (27%) and a tendency to increase in *P. dilatatum*.

Four major fatty acids were found in the leaves of the gramineae under study, namely palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) (Table 4.1.). However, their relative percentage in the control plants varies from species to species. Palmitic and linoleic acids content are similar among species (around 17-20% and 16-22% of total fatty acids, respectively). Oleic acid contributes more to fatty acid composition in *C. dactylon* (around 34% of total fatty acids) and less in *Z. japonica* (around 9% of total fatty acids). On the contrary, in the latter species leaf fatty acids presented a higher contribution of linolenic acid (53% of total fatty acids) than *C. dactylon* and *P. dilatatum* (respectively, 26% and 41% of total fatty acids). Accordingly, *Z. japonica* control plants presented the highest DBI values, while *C. dactylon* control plants the lowest (Table 4.1.).

One night-chilling tended to increase the unsaturated and to decrease the saturated fatty acid content in all species (Table 4.1.), however this tendency was not always

significant. The higher differences were obtained for *P. dilatatum*, where one night-chilled plants showed a decrease in palmitic acid that was accompanied by a similar increase in linoleic acid (a 22% change). However, the DBI was not altered after one night of chilling in any of the species under study.

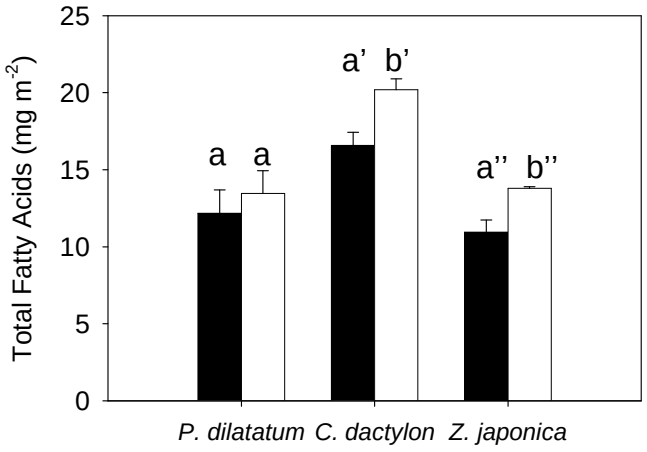

Figure 4.3. Leaf total fatty acid content for control (black bars) and one night-chilled (white bars) plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. Data are mean ± SD of three plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at P<0.05.

4.4.3. Electrolyte leakage and lipid peroxidation

Electrolyte leakage measurements showed that *Z. japonica* was the species that presented the highest value of conductivity (membrane leakage) in the control (around 55%), followed by *C. dactylon* (14%) and *P. dilatatum* (9%) (Table 4.2.). After one night-chilling the membrane leakage decreased by 23%, 19% and 58%, respectively, in *P. dilatatum*, *C. dactylon* and *Z. japonica*.

Zoysia japonica control plants showed almost twice the MDA content (around 11 μmol m^{-2}) that of *C. dactylon*, while it was five times higher than the lipid peroxidation products in *P. dilatatum* (Table 4.2.). After one night of chilling the lipid peroxidation was stimulated in *P. dilatatum* and *C. dactylon* (26% and 18% increase, respectively). In *Z. japonica* there was a decrease of about 21% in MDA content after stress imposition.

Table 4.1. Leaf fatty acid composition (% of total fatty acids) and double band index (DBI) for control and one night-chilled plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. Data are mean ± SD of three plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at P<0.05.

	C16:0 (%)	C18:1 (%)	C18:2 (%)	C18:3 (%)	DBI
P. dilatatum					
Control	17.2 ± 1.81 a	23.5 ± 2.19 a	18.5 ± 0.86 a	40.7 ± 1.72 a	183 ± 3.3 a
1 Night-Chilling	13.5 ± 1.78 b	22.2 ± 0.78 a	22.6 ± 2.00 b	41.7 ± 4.53 a	193 ± 8.8 a
C. dactylon					
Control	17.3 ± 0.85 a'	34.4 ± 3.88 a'	22.1 ± 0.35 a'	26.2 ± 3.68 a'	157 ± 7.3 a'
1 Night-Chilling	16.2 ± 3.06 a'	33.4 ± 1.71 a'	22.6 ± 2.82 a'	27.8 ± 1.27 a'	162 ± 4.4 a'
Z. japonica					
Control	21.2 ± 4.13 a''	9.4 ± 0.62 a''	16.6 ± 0.56 a''	52.7 ± 3.63 a''	201 ± 11.7 a''
1 Night-Chilling	16.9 ± 2.07 a''	7.3 ± 1.11 b''	17.2 ± 2.72 a''	58.6 ± 0.20 a''	217 ± 10.2 a''

4.4.4. Carboxylating enzyme activities

PEPC *Vphysiol* in control plants presented the highest values in *Z. japonica* (93 μmol m^{-2} s^{-1}) and the lowest in *P. dilatatum* (26 μmol m^{-2} s^{-1}) (Fig. 4.4.*a*,*b*,*c*). *Zoysia japonica* also presented the higher PEPC *Vmax* (152 μmol m^{-2} s^{-1}), while the lowest PEPC *Vmax* values were observed in *C. dactylon* (95 μmol m^{-2} s^{-1}). In all species the night-chilling treatment led to an increase in the PEPC *Vphysiol* (17% in *P. dilatatum* and *Z. japonica* and 31% in *C. dactylon*). PEPC *Vmax* was not affected by one night-chilling stress, except for *C. dactylon* where an increase was found (37%). In this species, PEPC activation state did not change after the short-term dark-chilling (Table 4.3.), whereas in *P. dilatatum* and *Z. japonica* it increased by 18% and 23%, respectively.

Rubisco *Vi* was higher in the control plants of *P. dilatatum* (11 μmol m^{-2} s^{-1}), followed by *Z. japonica* (8 μmol m^{-2} s^{-1}) and *C. dactylon* (4 μmol m^{-2} s^{-1}) (Fig.

4.4.*d*,*e*,*f*). The latter species also presented the lowest values of Rubisco *Vt*, while *Z. japonica* the highest ones. Dark-chilling affected Rubisco *Vi* and *Vt* differently among species. Rubisco *Vi* decreased 26% in *P. dilatatum*, increased 33% in *Z. japonica* and it was not affected in *C. dactylon*. On the contrary, Rubisco *Vt* did not change with stress in *P. dilatatum* and *Z. japonica*, but increased 28% in *C. dactylon*. Rubisco activation state (Table 4.3.) decreased after dark-chilling in *P. dilatatum* and *C. dactylon* (14% and 20%, respectively), but increased in *Z. japonica* (29%).

Table 4.2. Leaf conductivity values and malondialdehyde (MDA) equivalents for control and one night-chilled plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. Data are mean ± SD of six plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at P<0.05.

	Conductivity (%)	MDA equivalents ($\mu mol\ m^{-2}$)
P. dilatatum		
Control	9.1 ± 0.74 a	1.9 ± 0.11 a
1 Night-Chilling	7.0 ± 0.65 b	2.4 ± 0.26 b
C. dactylon		
Control	14.1 ± 2.72 a’	5.5 ± 0.75 a’
1 Night-Chilling	12.9 ± 2.14 b’	6.5 ± 0.55 b’
Z. japonica		
Control	55.1 ± 13.8 a’’	10.7 ± 1.13 a’’
1 Night-Chilling	23.0 ± 5.74 b’’	8.5 ± 0.54 b’’

4.5. Discussion

4.5.1. Dark-chilling stress led to an accumulation of carbohydrates and no changes in soluble protein content

To our knowledge there is no reference in the literature concerning the carbohydrates content in C_4 plants after being subjected to dark-chilling. There is, however, a study that shows a lower translocation of photoassimilates in dark-chilled C_4 species, *Echinochloa crus-galli* and *Eleusine indica*, linked to an increased turn-over

time of the export pools to the phloem (Potvin *et al.* 1985). This may explain the accumulation of both soluble (glucose, fructose and sucrose) and insoluble carbohydrates in leaves of the three C_4 species after one night of chilling (Fig. 4.1.). However, van Heerden *et al.* (2003*b*) showed no change in sucrose and starch content in two soybean genotypes after one to three nights of chilling although the photosynthetic rate decreased in the first night of stress in one of the genotype.

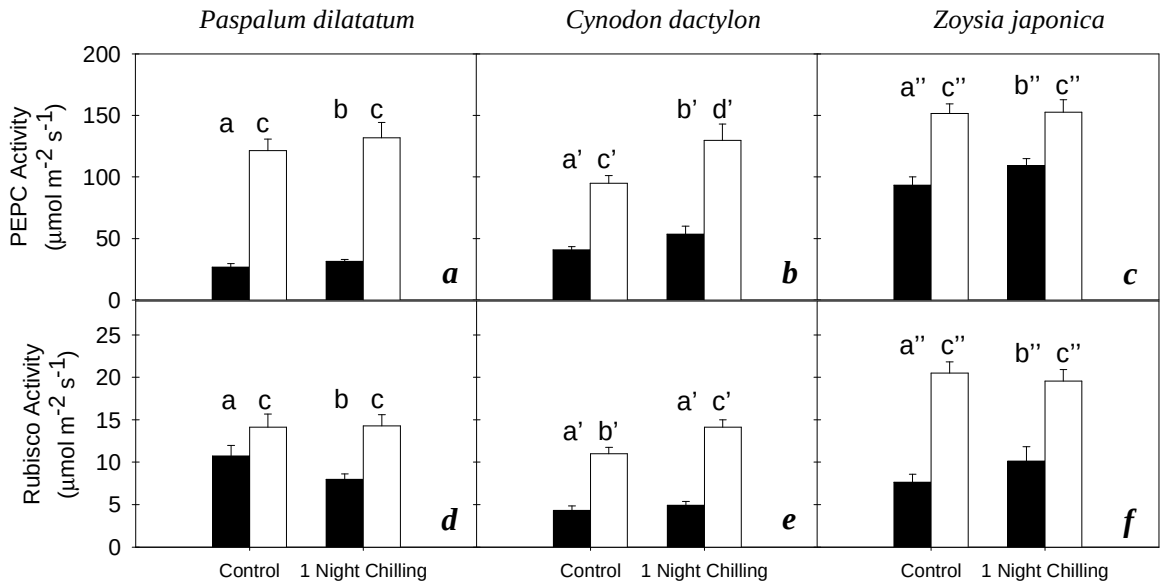

Figure 4.4. Phospho*enol*pyruvate carboxylase (PEPC) (**a**, **b**, **c**) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (**d**, **e**, **f**) activities *per* leaf area for control and one night-chilled plants of *Paspalum dilatatum* (**a**, **d**), *Cynodon dactylon* (**b**, **e**) and *Zoysia japonica* (**c**, **f**). Measurements of PEPC physiological (*Vphysiol*) and Rubisco initial (*Vi*) activities are represented in black bars, while white bars represent the activities of PEPC maximal (*Vmax*) and Rubisco total (*Vt*) activities. Carboxylating enzymes measurements were obtained from samples harvested inside the growth chamber at a PPFD of approximately 250-300 µmol m^{-2} s^{-1}. Data are mean ± SD of eight plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at $P<0.05$.

An increase in the carbohydrate content in leaves after dark-chilling may also reflect a lower metabolisation of sugars during the night period. A decrease in both cytochrome *c* oxidase and ATPase activities was found in maize seedlings after a short-term chilling stress (Prasad *et al.* 1994), decreasing the respiration rate.

The increase in the leaf carbohydrate content could contribute to decrease the dehydration degree of the cells, often observed under chilling and especially under freezing conditions (Bracale and Coraggio 2003). In addition to the function of

cryoprotection, sucrose accumulation could also provide a readily mobilized storage pool, which would be rapidly loaded into the translocation stream and used when needed upon return to more favourable conditions (Pollock and Lloyd 1987).

Table 4.3. Phospho*enol*pyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activation state for control and one night-chilled plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. Carboxylating enzymes measurements were obtained from samples harvested inside the growth chamber at a PPFD of approximately 250-300 μmol m^{-2} s^{-1}. Data are mean ± SD of eight plants of each species *per* treatment. The statistical analysis was performed separately for each species. The different letters represent statistical differences at P<0.05.

	PEPC activation state (%)	Rubisco activation state (%)
P. dilatatum		
Control	22 ± 2.0 a	70 ± 6.6 a
1 Night Chilling	26 ± 1.0 b	60 ± 5.1 b
C. dactylon		
Control	48 ± 4.7 a’	45 ± 5.2 a’
1 Night Chilling	46 ± 4.6 a’	36 ± 2.7 b’
Z. japonica		
Control	62 ± 4.3 a’’	38 ± 4.4 a’’
1 Night Chilling	76 ± 3.8 b’’	49 ± 6.1 b’’

It has been reported for many species that their ability to withstand cold maintaining the same rates of carbon assimilation is directly correlated with the increased ratio of soluble/insoluble carbohydrates (Hurry *et al.* 1995). This may be true for *P. dilatatum* and *Z. japonica* plants when photosynthesis was measured at a low light intensity (Chapter 3) similar to that of growth, but not for *C. dactylon*. In this species, the soluble/insoluble carbohydrates ratio did not change with dark-chilling, which may be related to the fact that *C. dactylon* presented a very high content of soluble carbohydrates in control conditions, especially glucose.

Although chilling injury is known to decrease protein synthesis and its turn-over in chilling-sensitive plants (Lyons 1973), our results show that a short-term dark-chilling stress for one night did not affect the soluble protein content in the three C_4 species

studied (Fig. 4.2.). However, the soluble protein content decreased in chilling-sensitive soybean plants subjected to three consecutive night-cold periods (Strauss *et al.* 2007) and in the cold-acclimated chilling-sensitive maize and semi-tolerant *P. dilatatum* plants (Cavaco 2000, Naidu *et al.* 2003), but increased in the cold-tolerant *Miscanthus* species (Naidu *et al.* 2003). Altogether these results suggest that chilling effect on soluble protein content depend on the chilling sensitivity of the species and on the duration and type of the imposed low temperature stress.

4.5.2. Leaf fatty acid unsaturation degree tended to increase and electrolyte leakage decreased after one night-chilling

As far as we know, changes in the leaf total fatty acid content and composition were never studied in response to a dark-chilling stress. However, it is well known that one of the first sites of chilling injury are cellular membranes (Lyons 1973; for review see Nishida and Murata 1996). A short-term chilling of 5ºC in the light after hardening of plants at 12ºC showed that the total fatty acid content of three chilling-sensitive species decreased rapidly, but remained barely constant in two chilling-tolerant species (Wilson and Crawford 1974). On the contrary, Welti *et al.* (2002) found an increase in the leaf total fatty acid content in *Arabidopsis thaliana* (Columbia ecotype) plants subjected to a short-term chilling stress during day and night. Our results show that leaf total fatty acid content after one night of chilling tend to increase in *P. dilatatum*, and was significantly higher in *C. dactylon* and *Z. japonica* (Fig. 4.3.). These results indicate that dark-chilling stress affected the turnover of fatty acids in the three species studied. The increase in total fatty acid content in *C. dactylon* and the tendency to increase in *P. dilatatum* may be related with an increase in lipid biosynthesis since in these two species the lipid peroxidation increased indicating a higher degradation of fatty acids (Table 4.2.). In *Z. japonica*, both an increase in fatty acid biosynthesis and a decrease in lipid peroxidation may have contributed to an increase in total leaf fatty acid content after dark-chilling.

The higher content of leaf fatty acids found in chilled plants may have contributed to the decrease in the leaf conductivity observed under these conditions (Table 4.2.). The decreased electrolyte leakage was very surprising since its increase is a well known process during chilling treatment in the light and in the dark (e.g. Creencia and

Bramlage 1971, Wright 1974, Patterson *et al.* 1976, Rosinger *et al.* 1984). Wright (1974) showed an increase in electrolyte leakage in common bean plants after 6 h in the dark at 5ºC, however it was observed a decrease of leaf conductivity up to control values 24 h after transferring the plants to light at 25ºC. Our results may indicate that before the full recovery of membrane integrity on the subsequent warm day after dark-chilling observed by Wright (1974), plants may initiate a shock response by increasing their membrane stability in the first hours at warm temperatures after the dark-chilling stress.

Our results also show that the four major fatty acids present in the leaves of these three gramineae are the palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) and the linolenic acid (C18:3) (Table 4.1.). These results are different from that of rhizomes, stolons and crowns of warm-season gramineae, as *Paspalum* (Cyril *et al.* 2001*a*) and *Cynodon* (Samala *et al.* 1998, Cyril *et al.* 2001*b*) species, in which stearic acid (C18:0) instead of oleic acid was present. This difference may result from the tissue being examined, since each organelle membrane, and thus each tissue, has a characteristic and distinct fatty acid composition and plant leaves generally present a higher content of oleic acid than stearic acid (for review see Ohlrogge and Browse 1995).

Paspalum dilatatum and *Z. japonica* are the species that presented the highest unsaturation degree (represented by the DBI) in the control plants (Table 4.1.), which is related to the highest content of linolenic acid, especially in the latter species. Although presenting the highest level of oleic acid, *C. dactylon* control plants showed the lowest degree of unsaturation due to its lower content of linolenic acid. Since it is generally accepted that a higher proportion of unsaturated fatty acids in control plants favours chilling tolerance (Murata *et al.* 1982), our results indicate that at membrane level, *Z. japonica* plants may be the less chilling-sensitive species, followed by *P. dilatatum* and *C. dactylon*. Dark-chilling tend to increase the percentage of leaf fatty acids with a higher unsaturation degree in all species, as commonly found in the literature for cold acclimation (for review see Nishida and Murata 1996). An increase in the proportion of unsaturated fatty acids, namely linolenic acid, seems to be higher in the chilling-tolerant than in the chilling-sensitive genotypes of maize (De Santis *et al.* 1999) and turf grass species, as *P. dilatatum* (Cyril *et al.* 2001*a*) and *C. dactylon* (Samala *et al.* 1998) exposed to cold acclimation.

As in Feng and Cao (2005) for tropical C_3 trees, an increase in ROS production after one night of chilling stress may have occurred at least in *P. dilatatum* and *C. dactylon*, since lipid peroxidation (approximated by MDA content) increased with cold in these species (Table 4.2.). An induction of oxidative stress and an effective up-regulation of the antioxidant metabolism has been observed in several C_3 species on the subsequent warm light period after a dark-chilling stress (Jun *et al.* 2001, van Heerden and Krüger 2002, Feng and Cao 2005), as in maize genotypes with a chilling treatment in the light (Foyer *et al.* 2002). The decrease in the lipid peroxidation in *Z. japonica* after dark-chilling indicates that an efficient antioxidant system occurred in this species, which may have also contributed to the higher decrease in electrolyte leakage, and thus its higher membrane integrity, compared with the other two C_4 species under study.

The higher lipid peroxidation found in *Z. japonica* control plants in relation to the other species (Table 4.2.) may be related to the higher proportion of linolenic acid, a fatty acid highly sensitive to peroxidation. In addition, a higher lipid susceptibility to peroxidation in control plants of this species can also explain its lower membrane integrity.

4.5.3. Carboxylating enzyme activities were altered by one night-chilling

Although several studies have reported a decrease in PEPC activity after a short-term exposure to low temperatures during day and night (e.g. Kingston-Smith *et al.* 1997), as far as we know there are no reports on this enzyme activity after a dark-chilling stress. Our results show that the C_4 species of different metabolic sub-types present a higher PEPC *Vphysiol* on the subsequent warming light period after one night-chilling (Fig. 4.4.). However, different strategies in relation to the increase in PEPC *Vphysiol* were found. In *P. dilatatum* and *Z. japonica* an increase in the enzyme activation state was observed, that may be related to a higher enzyme phosphorylation state (Table 4.3.). On the contrary, in *C. dactylon* the dark-chilling stress did not affect the enzyme activation state, since PEPC *Vphysiol* and *Vmax* presented a proportional increase. The PEPC *Vphysiol* increase in *C. dactylon* dark-chilled plants seem to result from an increase in the protein content, a similar result to that found by Bernardes da Silva *et al.* (1995) in *P. dilatatum* plants when acclimated to low temperature. The PEPC *Vmax* was also higher in the winter than in the spring months in *P. dilatatum*

grown under field conditions near Lisbon, where winter temperatures along the day were low and decreased significantly at night (Marques da Silva *et al.* 1990). The above results show that PEPC responses to low temperature depend on the type and duration of the stress and on the species studied.

Dark-chilling decreased Rubisco activation state in *P. dilatatum* and *C. dactylon* (Fig. 4.4. and Table 4.3.), by decreasing Rubisco *Vi* and increasing Rubisco *Vt*, respectively. The decrease in Rubisco *Vi* in *P. dilatatum* may be due to a lower carbamylation degree, while the increase in Rubisco *Vt* in *C. dactylon* may be related to an increase in enzyme content. On the contrary, in *Z. japonica* the activation state of Rubisco increased due to a higher *Vi*, indicating an increase in the carbamylation state. Martino-Catt and Ort (1992) showed that dark-chilling interrupted the circadian rhythm that regulates gene expression of Rubisco activase, an enzyme crucial for Rubisco carbamylation. The differences found in Rubisco carbamylation among the three C_4 species can be explained by a different sensitivity of Rubisco activase to dark-chilling, which may be related to a different ratio of enzyme polypeptides. Vargas-Suárez *et al.* (2004) showed a differential accumulation of the two Rubisco activase polypeptides (41 and 43 kDa) in maize leaves during leaf development and water stress, indicating functional biochemical significance for *in vivo* Rubisco activase subunits ratio. Different responses of Rubisco activity to chilling have also been observed in the literature. While in two night-chilled soybean plants Rubisco activity and activation state were not changed (van Heerden *et al.* 2003*b*), in the C_4 NAD-ME species *Muhlenbergia montana* (Nutt.) Rubisco activity decreased on the subsequent warm temperature and moderate light intensity after one night-chilling (Pittermann and Sage 2001). In addition, Rubisco activity in C_4 plants did not decrease after a short-term chilling in the light (Potvin *et al.* 1986). Du *et al.* (1999) showed that in *Saccharum* species the responses of several enzymes activity to low temperature for 4 h in the light depend on the species susceptibility to cold.

4.6. Conclusion

We conclude that the changes induced by dark-chilling in C_4 plants may involve alterations in the membrane flexibility, carbohydrate content and carboxylation enzyme

activities. Dark-chilling increased the carbohydrate content in the three C_4 species, although in a higher proportion in *P. dilatatum*. On the contrary, protein content was not affected by the imposed stress. A general tendency for an increase in fatty acid content was found, as well as in their unsaturation degree. Lipid peroxidation increased in *P. dilatatum* and *C. dactylon* dark-chilled plants. The higher unsaturation degree of *Z. japonica* control plants together with the higher increase in membrane integrity and the decrease in lipid peroxidation after the dark-chilling stress suggests that this species may be the least sensitive to low night temperatures at membrane level. Dark-chilling also altered the activation state of the carboxylating enzymes by different processes depending on the species studied. In general, the physiological or initial activity and the maximal or total activity, respectively for PEPC or Rubisco, increased or did not change after one night-chilling.

4.7. Acknowledgements

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4.8. References

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Chapter 5.

Regulation of photosynthesis and stomatal opening on each leaf surface with respect to light orientation in the C_4 species *Paspalum dilatatum*.

The results presented in this Chapter were obtained by Ana Sofia Soares at Rothamsted Research, Harpenden, UK except for the electron microscopy, which was performed at CEBAS-CSIC, Murcia, Spain by Dr. Enrique Olmos on samples prepared at Rothamsted Research by Ana Sofia Soares.

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- Soares AS, Driscoll SP, Olmos E, Arrabaça MC, Foyer CH (2008) Adaxial/abaxial specification in the regulation of photosynthetic CO_2 assimilation with respect to light orientation and growth with CO_2 enrichment in *Paspalum dilatatum* leaves. *New Phytologist* 177, 186-198.

- Soares AS, Driscoll SP, Olmos E, Harbinson J, Arrabaça MC, Foyer CH (2007) Adaxial/abaxial specification in the regulation of photosynthesis with respect to light orientation and growth with CO_2 enrichment in the C_4 species *Paspalum dilatatum*. In: Book of Abstracts (Photosynthesis Research, Vol 91, Nºs 2-3, 2007), 14th International Congress of Photosynthesis, SECC, Glasgow, Scotland, UK.

- Soares AS, Driscoll SP, Arrabaça MC, Foyer CH (2006) Differential regulation of stomata and photosynthesis on the adaxial and abaxial leaf surfaces of the C_4 species *Paspalum dilatatum* in response to CO_2 enrichment. In: Comparative Biochemistry and Physiology, Society for Experimental Biology (SEB) Annual Main Meeting 2006 Abstracts, University of Kent at Canterbury, Canterbury, England, UK, Volume 143/A, S173-S184.

5. Regulation of photosynthesis and stomatal opening on each leaf surface with respect to light orientation in the C_4 species *Paspalum dilatatum*.

5.1. Abstract

In this Chapter, the surface-dependent regulation of photosynthesis in the C_4 monocotyledonous species *Paspalum dilatatum* Poiret cv. Raki was studied. Gas-exchange measurements on the whole leaf and on each leaf surface separately were performed, with light entering the leaf either via the adaxial or the abaxial surface. The CO_2-response curves for photosynthesis on the abaxial surface were typical of C_4 leaves, saturating rapidly at low intercellular CO_2 (*Ci*) concentrations. Under these *Ci* concentrations the CO_2 assimilation rates were much lower on the adaxial surface, an effect that was most pronounced when light was oriented to the abaxial side. Illumination of the abaxial leaf surface also led to a complete cessation of photosynthesis and stomatal conductance on the adaxial surface, resulting in a lower whole leaf steady-state rate of photosynthesis in the CO_2-response curve. The asymmetric surface-specific regulation of photosynthesis and stomatal conductance observed with respect to light orientation was not caused by dorso-ventral variations in leaf structure, distribution of phospho*enol*pyruvate carboxylase and ribulose-1,5-bisphosphate carboxylase/oxygenase proteins or light absorptance, transmittance or reflectance. Adaxial/abaxial specification in the regulation of photosynthesis may result from differential sensitivity of stomatal opening to light and/or from different total surface area ratios of the bundle sheath cells and the surrounding first layer of mesophyll cells on each leaf side.

Keywords: abaxial surface, adaxial surface, bundle sheath, C_4 photosynthesis, leaf optical properties, mesophyll, monocotyledonous leaves, PEPC, Rubisco, stomatal conductance.

5.2. Introduction

Light orientation is a crucial regulator of the photosynthetic process (Poulson and DeLucia 1993), playing a crucial role in ecosystem sustainability (Millennium Ecosystem Assessment 2005). The interactions of stomatal function with light orientation are known since the 70's, being then showed a higher photosensitivity of the stomata on the abaxial than on the adaxial leaf surface in several C_3 and C_4 species with amphistomatous leaves (e.g. Turner 1970, Pemadasa 1979, Travis and Mansfield 1981). However, while the interactions of photosynthesis and light orientation have been widely studied in C_3 dicotyledonous leaves (e.g. Syvertsen and Cunningham 1979, Terashima and Inoue 1985*a*, Terashima 1986, Smith and Ulberg 1989), few studies exist on the effects of light orientation in monocotyledonous C_4 species.

Dicotyledonous C_3 plants present a different structure across the leaf and exhibit internal gradients in light and photosynthetic capacity (Terashima and Inoue 1985*a*, Terashima and Inoue 1985*b*). The relationship between leaf structure and function has been intensively studied in these plants especially in what concerns photosynthesis (Terashima and Saeki 1983, Cui *et al*. 1991, Vogelmann 1993). The highest photosynthesis rates are not found near the leaf surface where the light intensity is the highest, but are observed in the middle and lower palisade layers (Nishio *et al*. 1993, Evans 1995, Sun *et al*. 1998, Sun and Nishio 2001, Evans and Vogelmann 2003). It has been shown that the middle palisade cells have chloroplasts with higher electron transport activity, content of electron transport components, activity of PSI, PSII and ATPase, chlorophyll *a* to chlorophyll *b* ratio, amount of photosynthetic proteins (higher Rubisco to chlorophyll ratio) and less stacking of the thylakoids (Terashima and Inoue 1985*b*, Terashima and Evans 1988, Sun and Nishio 2001). Furthermore, the adaxial surface of dicotyledonous C_3 leaves has characteristics that resemble classic 'sun' leaves, while the abaxial surface has properties that are consistent with 'shade' leaves (Oya and Laisk 1976, Terashima 1986, Terashima and Evans 1988, Lambers *et al*. 1998).

In contrast to C_3 dicotyledonous species, the photosynthetic light-response curves of *Zea mays* L. (maize) are similar regarding the orientation of the leaf towards light (Moss 1964). However, it has been shown that in maize the photosynthetic CO_2-response curves of the adaxial and the abaxial leaf surfaces are different when light is

oriented to the adaxial surface (Domes 1971, Driscoll *et al*. 2006), indicating that although structurally similar the two leaf surfaces are photosynthetically different. Driscoll *et al.* (2006) showed that CO_2-response curve of photosynthesis on the adaxial surface is specifically determined by growth CO_2 abundance and tracks transpiration, while the photosynthesis on the abaxial surface is largely independent of CO_2 concentration and rather independent of stomatal function.

The present study was undertaken in order to characterize the relative effects of light orientation towards the leaf on photosynthesis and stomatal responses in *Paspalum dilatatum*, which is, like maize, a C_4 monocotyledonous species of the NADP-malic enzyme (NADP-ME) subtype. Plants were grown at ambient CO_2 concentrations and CO_2- and light-response curves of photosynthesis and stomatal conductance were performed both on the whole leaf and on each surface separately, with light oriented to the adaxial or the abaxial surface. In order to characterize the two leaf surfaces, the epidermal structure, the optical properties and the ratio of the total surface area of the bundle sheath cells and the surrounding first layer of mesophyll cells were studied on the adaxial and abaxial leaf surface, as well as the distribution of the carboxylating enzymes phospho*enol*pyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco).

5.3. Material and Methods

5.3.1. Plant material and growth conditions

Seeds of *Paspalum dilatatum* Poiret cv. Raki (NADP-malic enzyme, NADP-ME) were germinated on compost containing slow-release fertilizer in pots (20 cm diameter) and then grown for 6 weeks in a controlled-environment cabinet (Sanyo SGC228.CFX.J, Sanyo, Osaka, Japan) under a 16 h photoperiod, 25/19°C day/night temperature regime, CO_2 concentration of 350 ± 20 μL L^{-1} and 80% relative humidity. The CO_2 was supplied from a bulk container via a Vaisala GMT220 CO_2 transmitter (Vaisala Oyj, Helsinki, Finland) and was controlled using a Eurotherm 2704 controller (Eurotherm Ltd, Worthing, UK). A photosynthetic photon flux density (PPFD) of 600–650 μmol m^{-2} s^{-1} (400–700 nm) at pot height was provided by Philips Master TL5 HO

49w/830 fluorescent lamps (Philips Lighting UK, Guildford, UK). All plants were well watered daily.

Experiments were performed from the first to the fifth hour after the beginning of the light period on the middle and widest part of fully expanded leaves.

5.3.2. Gas-exchange measurements

Photosynthetic gas-exchange measurements were performed using an infrared gas analyser (model wa-225-mk3, ADC, Hoddesdon, UK). Two different types of chambers were used in these studies. A series of standard Parkinson chambers for the whole leaf measurements (Novitskaya *et al*. 2002) and a series of modified Parkinson chambers (Fig. 5.1.) for separate and simultaneous measurements of gas exchange on each leaf surface (Driscoll *et al*. 2006) were used.

To check the degree of CO_2 transport across the leaf, a gas stream containing 10% CO_2 was applied to each surface independently inside the chamber and the concentration on the other surface was measured. In this system, we could detect no passage of CO_2 from one surface to the other through the leaf blade. Although the stomata close under these conditions, this simple experiment illustrates that there is no passage of gases from one side of the chamber to the other. Each half of this dual chamber has a distinct gas supply and analysis unit with separate humidity, leaf temperature and individual fan controls (Fig. 5.1.*a*,*b*,*c*), operating as a whole leaf chamber. Flow rates were optimized on each side of the chamber to match the pressure on both leaf surfaces. Because the *P. dilatatum* leaf was not large enough to fully separate the two halves of the chamber, the leaf was expanded with gas-tight tape to seal each side of the chamber, preventing any flux of gas between them (Fig. 5.1.*d*,*e*). Light was supplied only from the top of the chamber as described previously (Novitskaya *et al*. 2002). Leaf temperatures were monitored via thermocouples attached to each surface separately (Fig. 5.1.*b*) and were maintained at 20°C on both the adaxial and abaxial surfaces, by water jackets, in order to ensure that the illuminated surface had exactly the same temperature as the unilluminated surface. All experiments were conducted at 50% relative humidity. The gas composition was controlled by gas mixers supplying CO_2 at concentrations as indicated in the figures with 21% O_2 and balance N_2.

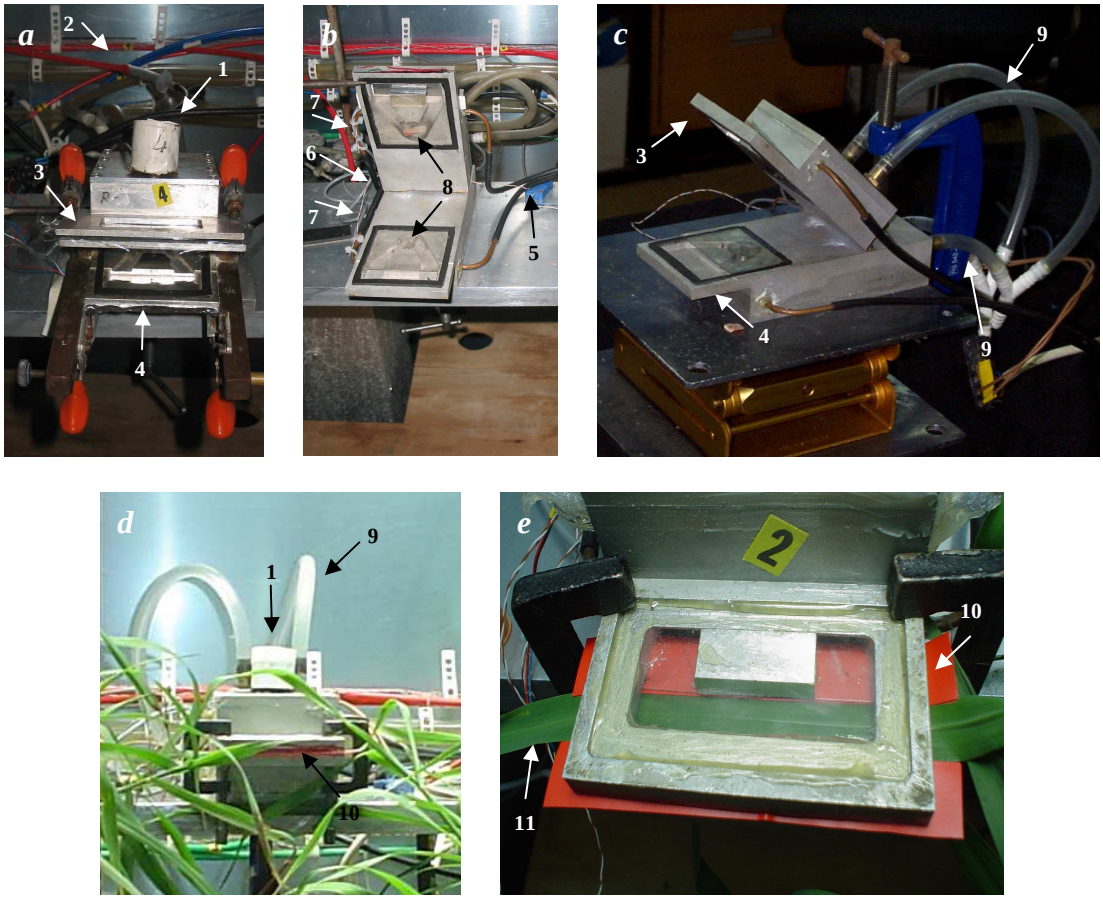

Figure 5.1. The modified Parkinson leaf chamber used for separate and simultaneous gas exchange measurements on each leaf surface. Different views of the chamber are given to illustrate the following features: 1, light sensor; 2, connecting tubes to the infrared gas analyser; 3, upper side of the modified chamber with light sensor; 4, lower side of the modified chamber; 5, air supply into both sides of the chamber; 6, air supply out of both sides of the chamber; 7, humidity and temperature sensors on both sides of the chamber; 8, fan on both sides of the chamber; 9, water jackets on both sides of the chamber; 10, gas-tight tape; 11, *Paspalum dilatatum* leaf.

Net CO_2 assimilation rate (*A*) and stomatal conductance to water vapour (*gs*) were obtained from CO_2- and light-response curves for the whole leaf and each leaf surface separately. Light was oriented directly either to the adaxial surface or to the abaxial surface (by inverting the leaf in the chamber). For CO_2-response curves, CO_2 was increased step-wise from 50 to 1000 μL L^{-1} at an irradiance of 900-1000 μmol m^{-2} s^{-1}. Light-response curves for photosynthesis were obtained via step-wise increases in irradiance from darkness to 1500 μmol m^{-2} s^{-1} at 360 μL L^{-1} CO_2. Steady-state gas-exchange measurements were obtained for each treatment after at least 10 min incubation under each light or CO_2 condition. Vapour water deficit was kept constant throughout each curve assay in both types of analysis.

Each value presented is the mean of nine plants.

5.3.3. Leaf optical properties

Leaf reflectance and transmittance measurements were performed across the spectrum from 400 to 800 nm at 1 nm intervals on each leaf surface of *P. dilatatum* plants. Reflectance and transmittance were measured using reflection and irradiance integration spheres (Avasphere-30 reflection and irradiance integration spheres, Avantes, Eerbeek, the Netherlands) connected to a USB-2000 spectroradiometer (Ocean Optics, Dunedin, Florida, USA). The measuring light was provided by a laboratory-built stabilized quartz-halogen source connected to a 0.6 mm optical fibre (type QP600-2-VIS-BX, Ocean Optics). In both cases the angle of incidence of the measuring beam was 8°. The 100% reflectance signal was obtained using a white reflectance standard (Spectralon; Labsphere, North Sutton, New Hampshire, USA). This was calibrated against a sheet of glass microfibre paper (Type GF/A, Whatman, Brentford, UK), which was used as a second standard. Leaf absorptance was calculated for each wavelength, as 1 – (reflectance + transmittance).

The experiment was performed independently in three plants. In each plant, five measurements on both the adaxial and abaxial sides of the leaf were obtained from leaf pieces parallel to the middle vein.

5.3.4. Epidermal structure

Leaf sections were painted on the adaxial and abaxial surfaces with clear nail varnish. Epidermal imprints were then stripped from both surfaces and examined by optical microscopy (Olympus BH-2, Olympus Optical Co. Ltd, Tokyo, Japan). The number of stomata was counted on 24 randomly selected digitized images from the adaxial and the abaxial epidermal imprints of eight plants. To calculate epidermal and stomatal cell areas and densities, the dimensions of at least 100 cells from the adaxial or the abaxial epidermal layers were measured using Sigma Scanpro photographic analysis software, version 5 (Sigma Chemical Co., St Louis, Missouri, USA). The stomatal index was calculated as the number of stomata/(number of epidermal cells + number of stomata) × 100, as defined by Salisbury (1927). The ratio of stomata between both

surfaces was calculated from the number of stomata counted on the adaxial and abaxial surfaces.

5.3.5. Fixation, embedding and sectioning for optical and electron microscopy

Leaf blade samples (1 mm^2) were fixed at ambient temperature in 4% (v/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.2) for 3.5 h. The samples were then washed in the same buffer three times for 15 min. They were then dehydrated at ambient temperatures using a graded ethanol series (35, 50, 70, 96 and twice at 100% (v/v)) with 30 min exposure at each concentration. The embedding was carried out in London Resin White acrylic resin (LRWhite, Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA) according to the following schedule: 75% (v/v) ethanol + 25% (v/v) LRWhite, 50% (v/v) ethanol + 50% (v/v) LRWhite, 25% (v/v) ethanol + 75% (v/v) LRWhite, 100% (v/v) LRWhite (1 h each series), and 100% (v/v) LRWhite overnight. The samples were transferred to tubes filled with resin and polymerized under nitrogen ambient at 55ºC for 24 h. Transverse semithin leaf sections (0.5 μm) for optical microscopy and transverse ultrathin leaf sections for electron microscopy (50–60 nm) were prepared using a Leica EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany). Optical microscopy sections were stained with toluidine blue. Electron microscopy sections were mounted on nickel grids and stained with lead citrate (Reynolds 1963) and 2% (w/v) uranyl acetate. Optical microscopy sections were observed using a Leica DMR light microscopy (Leica Microsystems, Wetzlar, Germany).

5.3.6. Surface area ratio of bundle sheath cells and the surrounding first layer of mesophyll cells *per* vascular bundle

The total surface area of the bundle sheath (BS) cells and the surrounding first layer of mesophyll (M) cells *per* vascular bundle on the adaxial and the abaxial leaf surface were obtained from transverse semi-thin leaf sections embedded in LRWhite resin. Measurements were obtained from photomicrographs taken with the same magnification using a Leica QM500 (Leica Microsystems, Wetzlar, Germany) imaging

analysis (Olmos *et al.* 2006) and correspond to the total surface area of bundle sheath cells and the surrounding first layer of mesophyll cells *per* vascular bundle. Each side of the leaf was separate taken into account the middle of the bundle sheath, which in general correspond to the middle of the leaf.

Each value is the mean of three plants, with a total of 25 different leaf vascular bundles observed from nine transverse semi-thin leaf sections (three plants x three sections).

5.3.7. *In situ* immunolocalization of Rubisco and PEPC

The nickel grids were incubated at ambient temperature for 30 min in phosphate-buffered saline (PBS; 173 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4.12H_2O$, 1.5 mM KH_2PO_4) containing 5% (w/v) bovine serum albumin (BSA). They were then incubated at room temperature for 3 h with either rabbit preimmune serum (dilution 1 : 500), rabbit anti-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (Agrisera AB, Vännäs, Sweden) (dilution 1 : 250) or rabbit anti-phospho*enol*pyruvate carboxylase (PEPC) (courtesy of Jean Vidal, Institut de Biotechnologie des Plantes, Centre National de la Recherche Scientifique, Université de Paris-Sud, France) (dilution 1 : 500), in the above PBS/BSA mixture. After washing twice with PBS (5 min each wash), the sections were incubated at ambient temperature for 1.5 h with goat antirabbit antibodies labelled with gold 10 nm (British Biocell International, Cardiff, UK) diluted in PBS containing 1% (w/v) BSA (dilution 1 : 50). The sections were washed sequentially with PBS containing 1% (w/v) BSA and then twice with PBS alone followed by five washes with filtered (0.2 μm) ultra-pure water (5 min each wash). The grids were dried at ambient temperature. Immunogold electron microscopy images were obtained with a Philips Tecnai 12 electron microscope (Philips, The Hague, the Netherlands) operated at 80 kV. The number of gold particles on the adaxial and abaxial side of the leaves, separately as explained on Section 5.3.6., were quantified using the 3.2 image analysis software (Soft Imaging System, Münster, Germany).

Each value is the mean of three plants, with a total of 45 different M and BS cells analysed on each side of the leaf.

5.3.7. Statistical analysis

The data were statistically analysed using parametric tests at a stringency of $P<0.05$. The significance of variation in mean values for epidermal structure, leaf BS and M cell area around each vascular bundle and immunological measurements was analysed for each parameter using a T-test.

5.4. Results

5.4.1. Leaf epidermal structure

The epidermal cells on the adaxial leaf surface were arranged in parallel rows with stomata in every third or fourth row (Fig. 5.2.*a*). In contrast, the epidermal cells on the abaxial surface were similarly arranged in parallel rows with stomata located in every second or third row (Fig. 5.2.*b*).

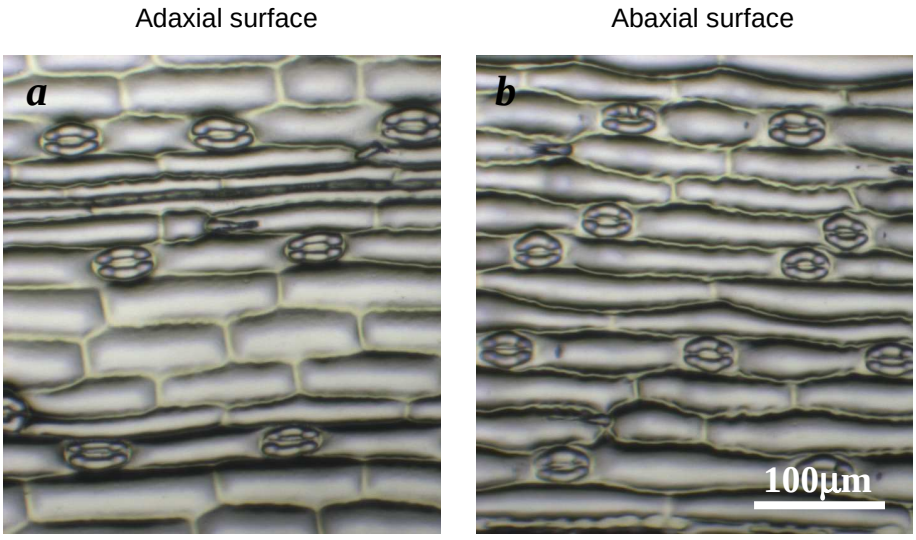

Figure 5.2. A comparison of the epidermal structure on the adaxial (***a***) and abaxial (***b***) leaf surfaces in *Paspalum dilatatum*. Bar, 100 µm.

The average epidermal cell area and number was comparable on both leaf surfaces (Table 5.1.). However, the average stomatal area and number did not follow the same

pattern on both leaf surfaces. While the average stomatal area is higher on the adaxial surface, the average stomatal number was higher on the abaxial surface (Table 5.1.). As a result, the ratio of stomata on the adaxial to the abaxial surface was *c*. 0.7 (Fig. 5.2. and Table 5.1.). Furthermore, the stomatal index was higher on the abaxial surface (Table 5.1.).

Table 5.1. Epidermal structure parameters on the adaxial and abaxial leaf surfaces in *Paspalum dilatatum* plants. Data represent the average ± SE for eight plants, with a total of 24 different digitized images measured. The statistical analysis was performed separately for each parameter analysed. The different letters represent statistical differences at P<0.05.

Parameter	Adaxial surface	Abaxial surface
Epidermal cell area (μm^2)	2762 ± 172.1 a	2704 ± 146.4 a
Epidermal cells (number mm^{-2})	283 ± 8.0 a	294 ± 6.1 a
Stomatal cell area (μm^2)	743 ± 20.6 a	696 ± 17.1 b
Stomatal density (number mm^{-2})	62 ± 2.4 a	89 ± 1.0 b
Stomatal index	18 ± 0.4 a	23 ± 0.4 b
Ratio of stomata (adaxial/abaxial)	0.70	

5.4.2. Whole leaf photosynthesis and light absorptance, transmittance and reflectance

Whole leaf *A* increased as atmospheric CO_2 increased over a range of low intercellular CO_2 concentrations (*Ci*), in a similar way regardless of the light orientation towards the leaf (Fig. 5.3.*a*). However, steady-state rates of photosynthesis were slightly higher when light was oriented to the adaxial surface (Fig. 5.3.*a*). Whole leaf *gs* decreased with increasing *Ci* and showed similar trends whether light was supplied via the adaxial or abaxial surface of the leaves. However, the overall *gs* values were lower when irradiance entered the leaf via the adaxial surface (86%, Fig. 5.3.*c*).

The light-response curves of whole leaf photosynthesis were similar regardless of the light orientation towards the leaf (Fig. 5.3.*b*). The *gs* increased with increasing

irradiance whether light was supplied via the adaxial or abaxial surface (Fig. 5.3.*d*), but its value was higher when the light entered the leaf via the abaxial surface (Fig. 5.3.*d*).

Leaf absorptance, transmittance and reflectance profiles were similar on both leaf surfaces across the light spectrum from 400 to 800 nm, whether light was supplied via the adaxial (Fig. 5.4.*a*) or the abaxial (Fig. 5.4.*b*) surface.

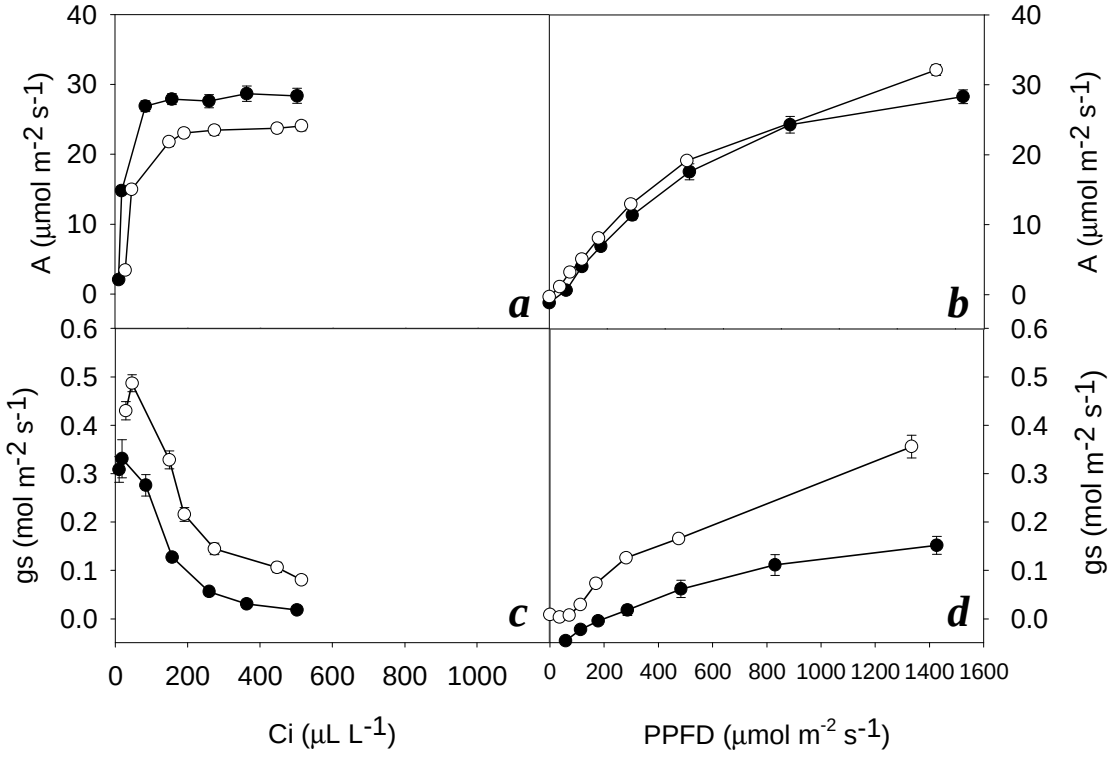

Figure 5.3. The effect of light orientation on the whole leaf net CO_2 assimilation rate (*A*) and stomatal conductance to water vapour (*gs*) in *Paspalum dilatatum* plants. The CO_2- (***a***, ***c***) and light- (***b***, ***d***) response curves for photosynthesis (***a***, ***b***) and stomatal conductance (***c***, ***d***) are presented. The light source was oriented either to the adaxial surface (closed circles) or to the abaxial surface (open circles). Data are the mean values ± SE of nine plants. Ci, intercellular CO_2 concentration; PPFD, photosynthetic photon flux density. The PPFD values correspond to leaf incident light intensity.

5.4.3. Photosynthesis in the adaxial and abaxial surfaces separately

The adaxial surface had much lower *A* values than the abaxial leaf surface at low *Ci* whether light entered the leaf via the adaxial or abaxial surface (Fig. 5.5.*a*,*b*).

Steady-state net CO_2 assimilation rates were similar on the two leaf surfaces when light was oriented to the adaxial side (Fig. 5.5.*a*). However, when light was oriented directly to the abaxial surface, steady-state rates of photosynthesis were much higher on

this surface (Fig. 5.5.*b*). In marked contrast, *A* values were almost null on the adaxial surface under these conditions (Fig. 5.5.*b*). When light was oriented to the adaxial surface, *gs* patterns decreased with increasing *Ci* similarly on both leaf sides (Fig. 5.5.*c*). However, when light was oriented to the abaxial surface (Fig. 5.5.*d*), higher *gs* values were found on this side of the leaf and almost no *gs* was detected on the adaxial surface.

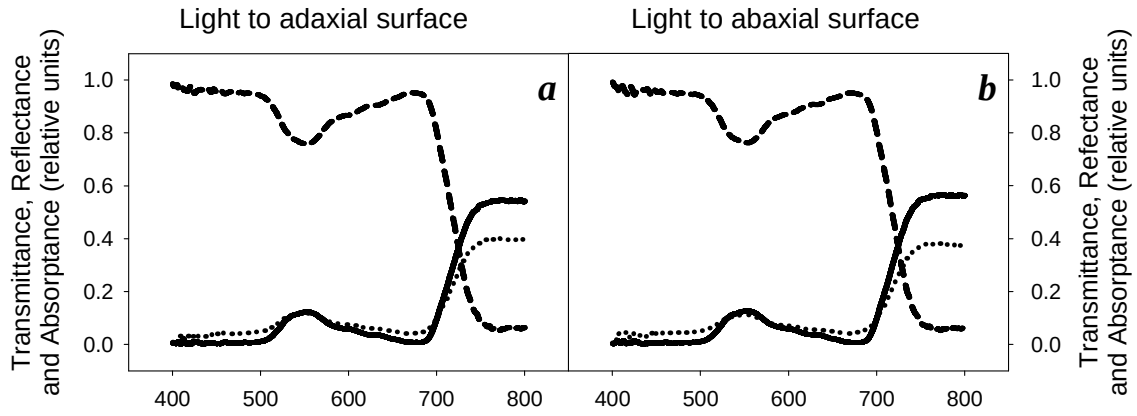

Figure 5.4. The effect of light orientation on the transmittance (solid line), reflectance (dotted line) and absorption (dashed line) spectra of *Paspalum dilatatum* leaves. The light source was oriented to either the adaxial (***a***) or the abaxial (***b***) surface. A single representative data set is shown.

When light was oriented to the adaxial surface, *A* increased with irradiance similarly on the two leaf surfaces (Fig. 5.6.*a*). In contrast, when light was oriented to the abaxial surface, photosynthetic rates on the adaxial side remained close to the compensation point regardless of the light intensity applied (Fig. 5.6.*b*). In marked contrast, the abaxial surface showed even higher CO_2 assimilation rates than that obtained when light was oriented to the adaxial surface (Fig. 5.6.*a*,*b*). Stomatal conductance values increased with light intensity and were similar on both leaf surfaces when light was oriented to the adaxial side of the leaf (Fig. 5.6.*c*). However, when light was oriented to the abaxial surface (Fig. 5.6.*d*), higher *gs* values were found on this side of the leaf when compared to the adaxial side where *gs* values were very low.

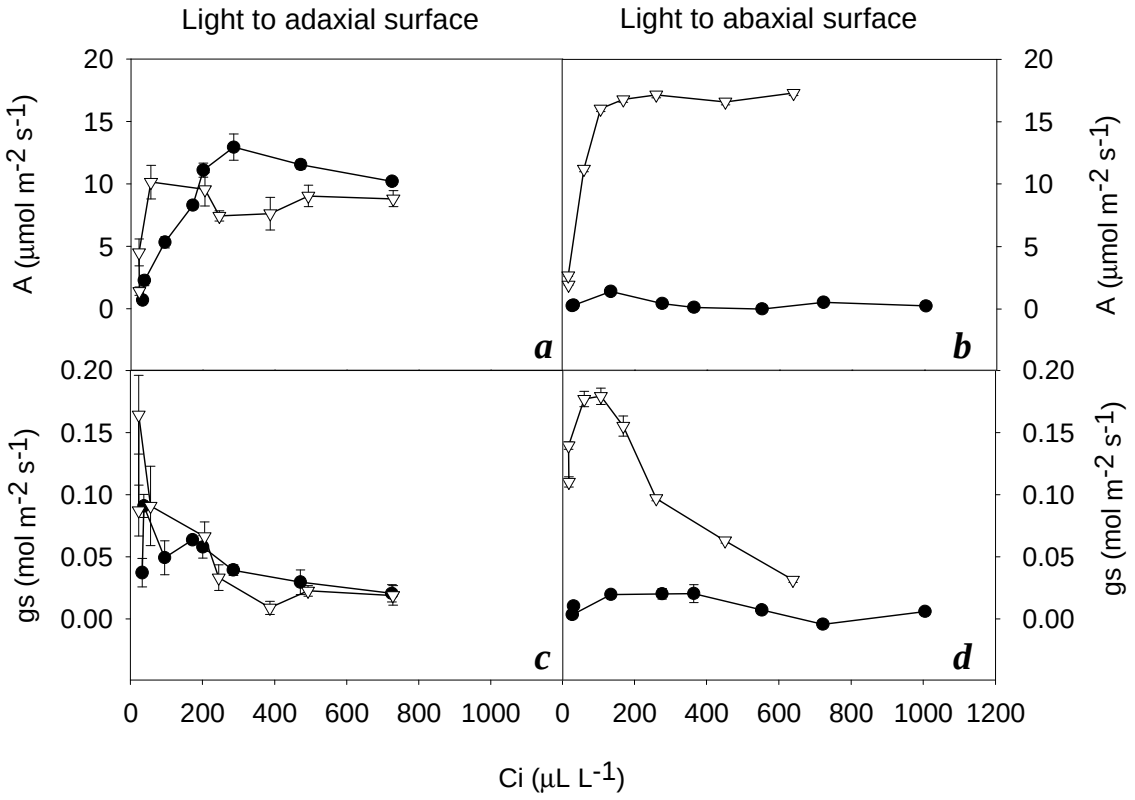

Figure 5.5. The effect of light orientation on the CO_2-response curves for net CO_2 assimilation rate (*A*, ***a***, ***b***) and stomatal conductance to water vapour (*gs*, ***c***, ***d***) on the adaxial (closed circles) and abaxial surfaces (open triangles) of *Paspalum dilatatum* leaves. The light source was oriented to either the adaxial (***a***, ***c***) or the abaxial (***b***, ***d***) surface. Data are the mean values ± SE of nine plants. Ci, intercellular CO_2 concentration.

5.4.4. Structure of the leaf vascular and mesophyll tissues

Paspalum dilatatum leaves have a single layer of BS cells surrounded by the M cells (Fig. 5.7.*a*). The BS generally has more cells on the abaxial than on the adaxial surface (Fig. 5.7.*b*). The mean value obtained for 50 different vascular bundles was 4.1 BS cells on the abaxial side and 3.0 BS cells on the adaxial side.

The total surface area of the bundle sheath cells *per* vascular bundle (BS cells area) is lower than the total surface area of the first layer of the mesophyll cells that surround the bundle sheath cells (M cells area, Table 5.2.). Furthermore, the BS and M cells area on each side of the *P. dilatatum* leaf is different. While the BS cells area is higher on the abaxial side, the M cells area is higher on the adaxial side. Accordingly, the ratio between the BS and M cells areas (BS/M area ratio) is lower on the adaxial side of the leaf.

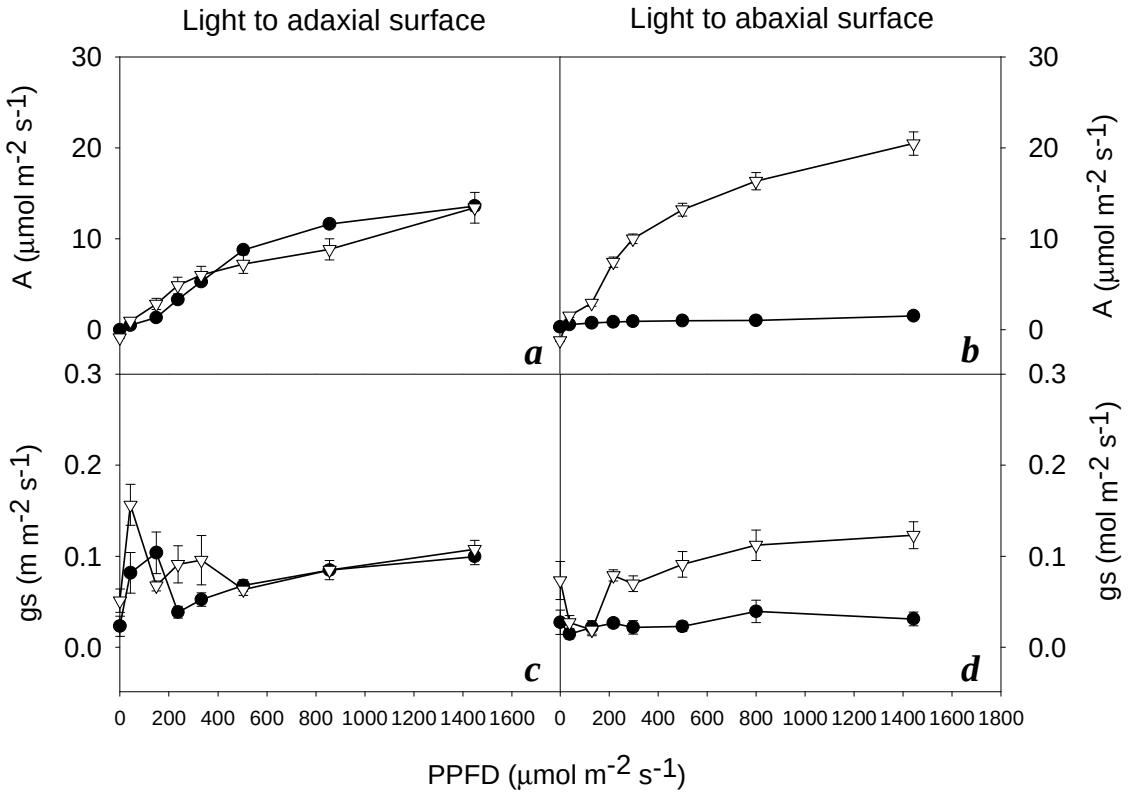

Figure 5.6. The effect of light orientation on the light-response curves for net CO_2 assimilation rate (*A*, ***a***, ***b***) and stomatal conductance to water vapour (*gs*, ***c***, ***d***) on the adaxial (closed circles) and abaxial surfaces (open triangles) of *Paspalum dilatatum* leaves. The light source was oriented to either the adaxial (***a***, ***c***) or the abaxial (***b***, ***d***) surface. Data are the mean values ± SE of nine plants. PPFD, photosynthetic photon flux density. The PPFD values correspond to leaf incident light intensity.

Regardless of the position in the leaf, the number of chloroplasts *per* cell ranged from three to eight in the BS and from four to seven in the M. However, the area occupied by chloroplast was much higher in the BS cells (27.9 ± 7.5 μm^2, ± standard deviation) than in the M cells (7.6 ± 1.8 μm^2), both on the adaxial and abaxial leaf surfaces.

5.4.5. *In situ* distribution of Rubisco and PEPC

In situ immunolocalization studies were performed on *P. dilatatum* leaves using specific antibodies against either the Rubisco large subunit (Fig. 5.7.*c*) or the PEPC protein (Fig. 5.7.*d*). This analysis revealed that these enzyme proteins were uniformly distributed across the leaf. No significant differences (P>0.05) in the amounts of

Rubisco or PEPC proteins were found in the adaxial and abaxial cells of the BS or M tissues across the leaf blade (Table 5.3.).

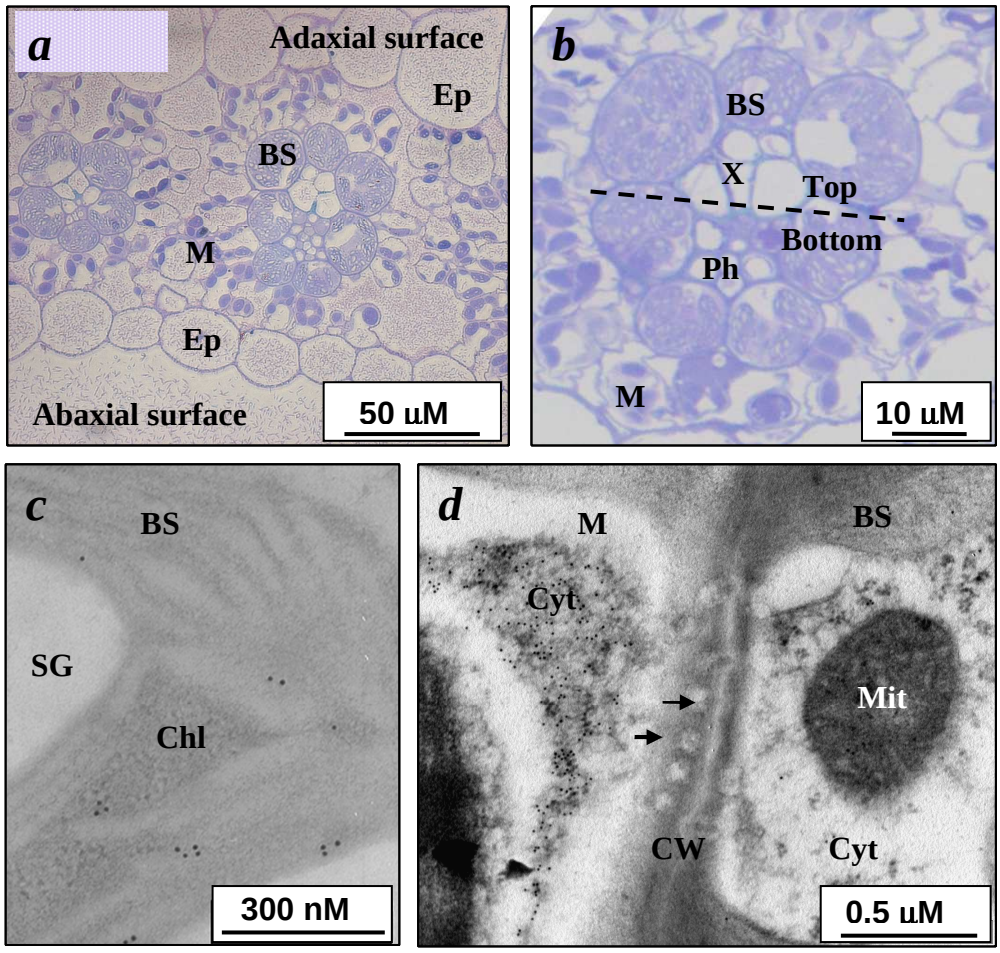

Figure 5.7. The structure of the *Paspalum dilatatum* leaf (***a***), its vascular bundle (***b***) and immunogold labelling of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (***c***) and phospho*enol*pyruvate carboxylase (PEPC) (***d***) proteins. BS, bundle sheath cell; Ep, epidermis; M, mesophyll cell; X, xylem; Ph, phloem; SG, starch grain; Chl, chloroplast; Cyt, cytoplasm; CW, cell wall; Mit, mitochondria. Dashed line indicates the zone of separation between the top and the bottom of the BS. Independent analyses for Rubisco and PEPC were performed. Bar, different sizes.

5.5. Discussion

5.5.1. The amphistomatous leaf of *P. dilatatum*

Stomata are the portals for gas exchange between the leaf mesophyll cells and the environment. While there is no difference concerning the epidermal cells, *P. dilatatum*

plants have amphistomatous leaves with 30% of the stomata on the adaxial and 70% on the abaxial surface (Fig. 5.2. and Table 5.1.). Amphystomy was proposed as an adaptation to reduce internal diffusion distance in thick leaves (Parkhurst 1978), as well as an adaptation to allow high stomatal conductances that are necessary to take advantage of high photosynthetic capacities (Mott *et al.* 1982). Amphystomy is also a rule in leaves of C_4 species, which have in general very high photosynthetic rates (for review see Mott *et al.* 1982). However, the ratio of stomata between the two leaf surfaces may vary with the light intensity at which plants are grown (Mott and Michaelson 1991) and with the leaf surface that is receiving light directly during the growth period (Aston 1978).

5.5.2. Whole leaf photosynthesis is not limited by light absorption under abaxial illumination

The CO_2-response curve of photosynthesis determined on the whole leaf showed a higher CO_2 saturating rate when the leaf is illuminate from the adaxial surface, indicating that *A* could be limited by light absorption with abaxial illumination of leaves (Fig. 5.3.). However, the whole leaf light-response curve of photosynthesis showed to be equal, regardless of the light orientation (Fig. 5.3.), as also observed by Moss (1964) in maize plants. Contrary to the results found for C_3 dicotyledonous species (e.g. Syvertsen and Cunningham 1979, Terashima 1986), the light-response curve results indicate that there are no differences in the absorption of light between the two leaf surfaces in this C_4 monocotyledonous species.

Table 5.2. Determination of the total surface area of the bundle sheath cells (BS cells area) and the total surface area of the surrounding first layer of the mesophyll cells (M cells area) *per* vascular bundle on the adaxial and abaxial sides of *Paspalum dilatatum* leaves. The ratio between the BS cells area and the M cells area (ratio BS/M area) is also presented. Data represent the mean values ± SE for three plants, with a total of 25 different vascular bundles measured. The statistical analysis was performed separately for each parameter analysed. The different letters represent statistical differences at $P<0.05$.

	Adaxial side	Abaxial side
Bundle Sheath Cells Area (μm^2)	1038 ± 40.6 a	1273 ± 71.5 b
Mesophyll Cells Area (μm^2)	2111 ± 87.6 a	1796 ± 90.4 b
Ratio Bundle Sheath/Mesophyll Area	0.50 ± 0.017 a	0.72 ± 0.029 b

The above suggestion was confirmed by the optical properties of the *P. dilatatum* leaf (Fig. 5.4.). We have shown that there were no differences between the two leaf surfaces in what concerns light absorption, reflectance or transmission, in accordance with the results obtained by Cordón and Lagorio (2007) for three monocotyledonous C_3 plants. Therefore, light-harvesting complexes and photosystems should be equally distributed on the adaxial and abaxial sides of the leaf in this monocotyledonous plant. This result is not surprising given the similarity of internal structure on the adaxial and abaxial sides of monocotyledonous leaves, which have a bilateral symmetry.

Table 5.3. Quantification of immunogold labelling for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phospho*enol*pyruvate carboxylase (PEPC) proteins in the adaxial and abaxial sides of *Paspalum dilatatum* leaves. Data represent the mean values ± SE for three plants, with a total of 45 different bundle sheath and mesophyll cells measured in each side of the leaf. Independent analyses for Rubisco and PEPC were performed. The statistical analysis was performed separately for each parameter analysed. The different letters represent statistical differences at $P<0.05$.

	Adaxial side (gold particles μm^{-2})	Abaxial side (gold particles μm^{-2})
Rubisco in chloroplasts		
Bundle Sheath Cells	15 ± 0.4 a	16 ± 0.4 a
Mesophyll Cells	3 ± 0.2 a	4 ± 0.2 a
PEPC in cytoplasm		
Bundle Sheath Cells	7 ± 0.4 a	8 ± 0.6 a
Mesophyll Cells	306 ± 7.8 a	333 ± 7.5 a

Although the similar levels of light penetration on the adaxial and abaxial leaf surfaces independently of leaf orientation towards light, it should be expected a gradient of light quantity and quality across the C_4 monocotyledonous leaf. Light absorption profiles in *Flaveria bidentis* and maize have shown that, while green light is absorbed throughout the leaf, blue light is only strongly absorbed near the surface with little penetration in the BS cells (JR Evans, TC Vogelmann and S von Caemmerer pers.

comm.). Since the adaxial and abaxial leaf surfaces of *P. dilatatum* have the same absorption profile (Fig. 5.4.), the gradient of light quantity and quality across the leaf should be the same regardless of the leaf surface that is being illuminated.

The CO_2- and light-response curves of the whole leaf *gs* indicate that the stomata respond differently to light orientation (Fig. 5.3.), being more open when light entered the leaf via the abaxial surface. This result will be discussed on Section 5.5.4.

5.5.3. Dorso-ventral asymmetry in the regulation of photosynthesis

Like in maize (Domes 1971, Driscoll *et al*. 2006), *P. dilatatum* leaves display a pronounced dorso-ventral asymmetry in the regulation of photosynthesis, with each surface showing unique characteristic responses to available CO_2 and light (Fig. 5.5, Fig. 5.6.). The gradients in CO_2 fixation rates that have been documented in dicotyledonous C_3 leaves (Oya and Laisk 1976, Terashima 1986, DeLucia *et al*. 1991, Sun and Nishio 2001, Evans and Vogelmann 2003) may also be present in this monocotyledonous C_4 leaves. The dorso-ventral gradients in photosynthetic machinery that determine adaxial/abaxial light absorption and CO_2 fixation characteristics in C_3 leaves must be entrained early in leaf development (Smith and Ullberg 1989, Evans *et al*. 1993, Poulson and DeLucia 1993, James and Bell 2000, Ustin *et al*. 2001) as they can only be changed by inversion of the leaves at the onset of development (Terashima *et al*. 1986, Smith *et al*. 1997). In addition, Driscoll *et al.* (2006) and Soares *et al.* (2008) showed that the dorso-ventrality of photosynthesis is maintained in maize and *P. dilatatum* plants grown under CO_2 enrichment, indicating that it must be genetically determined in these two monocotyledonous C_4 species.

A pertinent question concerns the underlying mechanism that determines the asymmetry in the dorso-ventral regulation of photosynthesis in the leaves of C_4 grasses since they show a structural dorso-ventral symmetry (Fig. 5.7.). As in dicotyledonous C_3 plants, the gradient in CO_2 assimilation across the leaf could depend on the quantity and quality of the light that reaches the illuminated and the non-illuminated surface, as well as on the distribution of organelles and enzymes across the leaf blade.

Although the light absorption by each leaf surface is similar in *P. dilatatum* (Fig. 5.4.), the quantity of light that reaches the illuminated surface must be higher than the one that reaches the non-illuminate surface. Under adaxial illumination, the lower light

quantity reaching the abaxial surface should have resulted in a lower photosynthetic rate on this surface in comparison to the adaxial one. However, the steady-state photosynthetic rates were similar on both leaf surfaces in the CO_2- (Fig. 5.5.) and light-response curves (Fig. 5.6.). These results indicate that, contrary to C_3 dicotyledonous, the abaxial side of the leaf in this monocotyledonous C_4 species may present a higher capacity to perform photosynthesis for the same light intensity. This was confirmed by the increase in the photosynthetic rates on the abaxial surface under abaxial illumination, both in the CO_2- and light-response curves (Fig. 5.5., Fig. 5.6.). In addition, Long *et al.* (1989) showed that under adaxial illumination the maize leaf needs more light intensity to saturate photosynthesis on the abaxial than on the adaxial surface, confirming the higher photosynthetic capacity of the former leaf surface.

Accordingly, a higher capacity for photosynthesis on the abaxial surface may also be related to a higher number of BS cells on this surface (Fig. 5.7.), which present a similar chloroplast number and area *per* BS cell to that of the adaxial surface. In addition, the higher number of BS cells and the higher BS/M area ratio (Table 5.2.) on the abaxial side of the leaf may allow a greater contact between BS and M cells. This higher contact would facilitate the metabolic transfer between the two cell types on the abaxial surface, allowing higher rates of photosynthesis to be reached on this surface under the same light intensity.

Furthermore, under adaxial illumination the initial slope of the CO_2-response curves, which represent the maximal PEPC rate according to von Caemmerer (2000), indicate a higher maximal PEPC activity on the abaxial surface. Since the immunolocalization studies show that there is a relatively uniform distribution of the carboxylating enzyme proteins (Rubisco and PEPC) across the *P. dilatatum* leaf (Table 5.3.), a different activation state of PEPC in the two leaf surfaces would explain the observations in the CO_2-response curve at low *Ci* (Fig. 5.5.). An increase in the PEPC activation state may involve an increase in the phosphorylation state of the enzyme protein (Chollet *et al*. 1996) and depends on several factors, namely the presence of the enzyme inhibitor L-malate and the effector glucose-6-phosphate. A higher activation state on the abaxial surface when the adaxial surface is being illuminated could imply, at least, an asymmetric gradient in the content of L-malate and/or glucose-6-phosphate.

The increase in photosynthesis on the abaxial leaf surface when the leaf is being illuminated through this surface (Fig. 5.5., Fig. 5.6.) is expected considering that the higher intensity of light reaching the abaxial leaf surface would increase the phosphorylation state of PEPC on this side of the leaf and thus its activity, as well as the activity of other processes involved in the photosynthetic metabolism. The decrease of photosynthesis on the adaxial leaf surface under abaxial illumination was expected due to the lower light intensity reaching this surface. However, the almost null values of photosynthesis (Fig. 5.5., Fig. 5.6.) were surprising and may be related to the almost closure of stomata on this surface. Since the method used to measure photosynthesis is based on the gas-exchanges between the chamber atmosphere and the leaf, if stomata are closed there is not exchange of gases and then no detectable photosynthesis.

Although CO_2 is not entering the leaf via the adaxial surface under abaxial illumination, this side of the leaf may be performing some photosynthesis due to the presence of internal CO_2 that may diffuse from the abaxial side of the leaf and a PEPC with a high-affinity for HCO_3^- (Bauwe 1986). The resistance to CO_2 diffusion through the mesophyll in the dicotyledonous species sunflower (Mott and O'Leary 1984) and cotton (Farquhar and Raschke 1978) has been shown to be low, and hence the CO_2 concentration differences within the leaf may be small. However, in the monocotyledonous species maize the resistance to CO_2 diffusion was higher than in cotton (Farquhar and Raschke 1978), what may be related to the close packing of cells in the former species. In addition, Long *et al.* (1989) showed the presence of two airspace systems in the mesophyll of maize leaves, one connected to the stomata on the adaxial epidermis and the other to the stomata on the abaxial epidermis, with few connections between them, suggesting that the resistance to CO_2 diffusion is higher in this monocotyledonous species.

5.5.4. Different sensitivity of stomata on the adaxial and abaxial leaf surface

The degree of stomatal opening was also markedly affected by light orientation in *P. dilatatum* leaves, both in the responses of the whole leaf (Fig. 5.3.) and of each leaf surface separately (Fig. 5.5., Fig. 5.6.). Under adaxial illumination the stomatal conductance was similar on the illuminated adaxial surface and on the non-illuminated

abaxial surface, indicating that the stomata on the abaxial surface require less light quantity to open. This was supported by the results obtained under abaxial illumination in which stomata almost closed on the non-illuminated adaxial surface whereas stomatal conductance increased on the illuminated abaxial surface (Fig. 5.5., Fig. 5.6.). Furthermore, the similar absorption, reflectance and transmittance profiles by each leaf surface (Fig. 5.4.), indicating that the quantity and the quality of light that reaches the non-illuminated surface when the other is being illuminated is the same, supports the idea that the adaxial surface stomata are less sensitive to light than the stomata on the abaxial side.

The higher sensitivity to light of stomata on the abaxial leaf side is a well documented feature in the literature for both monocotyledonous and dicotyledonous C_3 and C_4 species with amphistomatous leaves (e.g. Turner 1970, Pemadasa 1979, Pospísilová and Solárová 1980, Travis and Mansfield 1981, Yera *et al.* 1986, Lu *et al.* 1993, Goh *et al.* 1995). According to Pemadasa (1979) the higher photosensitivity of abaxial stomata may be regarded as an ecologically adaptation to prevent closing under natural conditions, in which the abaxial stomata normally experience much lower light intensity than the adaxial ones. The author also suggests that this adaptive advantage may be especially significant in plants with fewer stomata on the adaxial than on the abaxial epidermis, as is the case of *P. dilatatum* leaves.

Although little is known about the physiological and biochemical properties for the different sensitivity of stomata to light, the literature results suggest that the adaxial and abaxial guard cells may present different signal transduction systems or pathways (Wang *et al.* 1998). In general, stomata open when an increase in the osmotic concentration of the guard cell drives water uptake and guard cell swelling. This increase in osmotic concentration results from the uptake of potassium and chloride ions and from the synthesis of malate involving starch breakdown (Assmann ans Shimazaki 1999). Ions uptake depends on the presence of a proton gradient between the guard cells cytoplasm and the apoplast, requiring the extrusion of protons from the guard cells (Roelfsema and Hedrich 2005). Pemadasa (1979) suggested that the different behaviour of the abaxial and adaxial stomata with the same light intensity is a reflection of different starch and potassium content in adaxial and abaxial guard cells when the stomata are open. In this case the abaxial guard cell presented a lower starch and higher potassium content. In addition, Goh *et al.* (1995) showed that the H^+-pumping activity

leading to the extrusion of protons from the guard cells on the adaxial epidermis required much higher light intensity than on the abaxial epidermis to reach the same activity level.

The higher stomatal conductance on the abaxial than on the adaxial leaf surface when receiving the same light intensity, i.e. respectively under abaxial or adaxial illumination, could also be a result of a higher stomatal number on the abaxial surface (Table 5.1.).

5.6. Conclusion

The dorso-ventral specification in the regulation of photosynthesis in *P. dilatatum* is not related with differences in leaf structure, carboxylating enzyme distribution and leaf optical properties across the leaf. It may, however, arises from differences in stomatal sensitivity to light orientation and to a different total surface area ratio between the BS cells and the surrounding first layer of M cells *per* vascular bundle on each leaf surface. Furthermore, it is suggested that a fixed gradient in enzyme activation, namely PEPC activation state, may also be present through the leaf. In addition to photosynthesis, stomatal conductance is differently regulated on each leaf surface, with the abaxial stomata showing a higher sensitivity to light. The results presented here, together with those reported previously in maize (Driscoll *et al*. 2006), suggest that dorso-ventral asymmetry in the regulation of photosynthesis and stomatal conductance may be a common feature of monocotyledonous C_4 species, at least from the NADP-ME subtype.

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5.8. References

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Chapter 6.
Dark-chilling effects on the surface-specific regulation of photosynthesis and stomatal conductance in the C_4 species *Paspalum dilatatum*.

The results presented in this Chapter were obtained by Ana Sofia Soares at Rothamsted Research, Harpenden, UK. A paper is being prepared to be submitted to a peer-reviewed journal.

6. Dark-chilling effects on the surface-specific regulation of photosynthesis and stomatal conductance in the C_4 species *Paspalum dilatatum*.

6.1. Abstract

The effects of chilling on the surface-specific regulation of photosynthesis in monocotyledonous C_4 species have never been described. In this Chapter we aimed to characterize the effects of one and two nights of chilling on the dorso-ventral regulation of net CO_2 assimilation rate and stomatal conductance responses in *Paspalum dilatatum*, a NADP-ME C_4 species. The plants were analysed regarding the pigment and soluble protein content and the photosynthesis on the whole leaf and on each leaf surface separately. The gas-exchange measurements were performed with light entering the leaf via the adaxial or the abaxial surface. While the total leaf pigment content decreased after the dark-chilling, the soluble protein content was not affected. The photosynthetic light-response curve on the whole leaf and on each leaf surface separately was not affected by dark-chilling regardless of light orientation. However, the whole leaf steady-state rate of photosynthesis measured at different CO_2 intercellular concentrations decreased more in one than in two nights-chilled plants under adaxial illumination. Furthermore, under this light orientation photosynthesis after one night-chilling tends to be more affected on the adaxial surface. Under abaxial illumination the photosynthesis on the whole leaf, as indicated by the parameters predicted from the application of the von Caemmerer's C_4 photosynthetic models, and on each leaf surface separately was little affected after dark-chilling. Results show that while the regulation of photosynthesis on the adaxial and abaxial leaf surfaces seems to be differently affected by dark-chilling in the monocotyledonous C_4 species *P. dilatatum*, stomatal conductance seems to be similarly affected on each leaf surface.

Keywords: abaxial surface, adaxial surface, C_4 photosynthesis, monocotyledonous leaves, one night-chilling, pigments, protein, stomatal conductance, two nights-chilling.

6.2. Introduction

In Chapter 5 we have shown that in the C_4 monocotyledonous species *P. dilatatum* the photosynthetic rate and the stomatal conductance show a leaf surface-specific regulation. As far we know few studies have been performed in order to understand if the photosynthetic and stomatal dorso-ventral regulation is altered in response to fluctuating environmental conditions.

It has been shown that in amphistomatous leaves of C_3 and C_4 dicotyledonous and monocotyledonous species the stomata on the adaxial epidermis are usually less sensitive to water stress (Turner and Singh 1984, Lu 1988, Wang *et al.* 1998), an osmotic stress as low temperature. The different responses of stomata on the adaxial and abaxial surfaces may be related to a different concentration of calcium ions on both surfaces guard cells (De Silva *et al.* 1986). The lower sensitivity of adaxial stomata to water stress is in accordance with the lower sensitivity of the guard cells on this surface to an increase in the concentration of ABA (abscisic acid) and calcium ions (Wang *et al.* 1998), molecules that controls the water stress response of guard cells (Vavasseur and Raghavendra 2005). However, stomatal conductance was showed to be more affected on the adaxial surface in the dicotyledonous C_3 species *Rumex obtusifolius* grown under CO_2 enrichment (Pearson *et al.* 1995). Although little is known about the intercellular second messengers involved in the stomatal response to CO_2 (Vavasseur and Raghavendra 2005), it is believed that the ABA and the CO_2 signalling pathways inducing stomatal closure may present some differences that could justify the different results obtained with the imposition of both stresses.

Regarding photosynthesis, Driscoll *et al*. (2006) showed that growth at high CO_2 alters the regulation of photosynthesis on the adaxial and abaxial leaf surfaces of the C_4 monocotyledonous species *Zea mays* (maize) in response to CO_2 availability, without affecting the dorso-ventrality of the leaf. They also show that the adaxial leaf surface photosynthesis is more affected by CO_2 enrichment. In *P. dilatatum* plants grown under CO_2 enrichment, photosynthesis either in response to CO_2 or to light availability decreased more on the adaxial than on the abaxial leaf surface (Soares *et al.* 2008).

To our knowledge no information is available on the effects of low temperatures on the surface-specific regulation of photosynthesis and stomatal conductance in monocotyledonous C_4 species, namely after a dark-chilling. However, low night

temperatures have been shown to decrease whole leaf photosynthesis and stomatal conductance on the subsequent warm day both in C_3 (e.g. van Heerden *et al.* 2003*a*, Flexas *et al.* 1999, Feng and Cao 2005) and C_4 (e.g. Ludlow and Wilson 1971, Pasternak and Wilson 1972, Pittermann and Sage 2001) species, in a manner that depends on the species sensitivity to cold, the duration of the stress and the light intensity at which plants are subjected on the following morning.

The present study was undertaken in order to characterize effects of a short-term dark-chilling on the surface-specific regulation of photosynthetic rate and stomatal conductance in the C_4 species *Paspalum dilatatum* (NADP-malic enzyme, NADP-ME). Plants were grown at ambient CO_2 concentrations and CO_2- and light-response curves of photosynthesis were performed for the whole leaf and each leaf surface separately, with light oriented to the adaxial or the abaxial surface. The whole leaf response curves allowed the determination of several parameters based on the whole leaf C_4 photosynthetic models described by von Caemmerer (2000). The dark-chilling effect on the whole leaf pigment and protein content was also determined.

6.3. Material and Methods

6.3.1. Plant material, growth conditions and dark-chilling imposition

Paspalum dilatatum plants were grown as referred in Chapter 5. The dark-chilling stress was imposed by transferring the 6 weeks old plants from the controlled growth chamber (25±19ºC) to a similar one at a night temperature of 4ºC. Plants were analysed on the subsequent light period after one and two nights of cold. Assays were performed from the first to the fifth hour after the beginning of the light period using the middle and widest part of fully expanded leaves.

6.3.2. Determination of pigments and protein content

Leaf pigments (chlorophyll *a* (chl*a*), chl*b*, total chlorophyll and carotenoids) and soluble protein contents were determined in the leaves of control and night-chilled *P. dilatatum* plants. Leaf sections (4 cm), excised from both sides of each leaf, parallel to

the middle vein, were ground in liquid nitrogen and quartz sand. Pigments were determined in the leaf extracts according to the method of Lichtenhaler and Wellburn (1983) in ethanol (96%, v/v). Total leaf soluble proteins were extracted in 1 mM sodium phosphate buffer (pH 7.0) containing 10 mM DTT, 1 mM PMSF, 5 mM 2-mercaptoethanol and 1% (w/v) Polyclar AT. Protein content was determined according to the method of Bradford (1976), using bovine serum albumin (BSA) as standard.

Each value presented corresponds to the mean of seven plants *per* treatment.

6.3.3. Gas-exchange measurements

Whole leaf, adaxial and abaxial leaf surfaces net CO_2 assimilation rate (*A*) and stomatal conductance to water vapour (*gs*) measurements for control and chilled plants were performed as referred in Chapter 5. Both CO_2- and light-response curves were obtained by illuminating the leaf via the adaxial or the abaxial surface (by inverting the leaf in the chamber). For CO_2-response curves the CO_2 concentration was increased step-wise from 50 to 1000 μL L^{-1} at an irradiance of 900-1000 μmol m^{-2} s^{-1}. The high light intensity used to obtain the photosynthetic CO_2-response curves was chosen to ensure that differences in the whole leaf net CO_2 assimilation rate could be found when *P. dilatatum* plants were dark-chilled (see Chapter 3) and to allow the comparison between this Chapter results and those of Chapter 5. The light-response curves for photosynthesis were performed via step-wise increases in irradiance from darkness to 1500 μmol m^{-2} s^{-1} at 360 μL L^{-1} CO_2. Steady-state gas-exchange measurements for each treatment were determined at 20ºC and after at least 10 min incubation at each light intensity or CO_2 concentration. Vapour water deficit was kept constant throughout the assay in both types of analysis.

Each curve presented corresponds to the mean of three plants *per* treatment.

6.3.4. C_4 photosynthetic model predictions for whole leaf photosynthesis

C_4 photosynthetic models were applied to the CO_2- and light-response curves. However, since the C_4 photosynthetic models were only described for the whole leaf photosynthesis it was decided not to apply the models to the adaxial and abaxial photosynthetic response curves. Furthermore, some difficulties could arise at least in the

interpretation of the maximal electron transport rate and maximal photosynthetic rate values obtained for each leaf surface (S von Caemmerer pers. comm.) since we do not know how much light reaches the non-illuminated surface.

6.3.4.1. CO_2-response curves

The whole leaf CO_2-response curves were used to predict the C_4 photosynthetic parameters, accordingly to the whole leaf C_4 photosynthetic model described by von Caemmerer (2000). The model allows the prediction of the maximal phospho*enol*pyruvate carboxylase (PEPC) carboxylation rate (*Vpmax*) and maximal ribulose-1,5-bisphosphate (Rubisco) carboxylation rate (*Vcmax*) using the enzyme-limited photosynthesis equations and the maximal electron transport rate (*Jmax*) using the light- and electron-transport-limited photosynthesis equations.

The model requires that the light intensity used to perform the curves is saturating. However, the light intensity we used (900-1000 μmol m^{-2} s^{-1}) was not fully saturating accordingly to Fig. 6.2., in order to avoid photoinhibition when plants were dark-chilled. Thus, it is important to retain that the values obtained through the application of the C_4 photosynthetic model are not real maximal values, and can only be used to compare the effects of dark-chilling and light orientation to the leaf.

In addition, the C_4 photosynthetic model requires the use of some parameters, which are tabled at 25ºC (von Caemmerer 2000). Some of these parameters are dependent on temperature and need to be transformed to the temperature at which the curves were performed (20ºC), as is the case of the Michaelis-Menten constants of Rubisco for CO_2 (K_c) and for O_2 (K_o) and the Michaelis-Menten constant of PEPC for CO_2 (K_p). To transform the K_c, K_o and K_p tabled values to 20ºC, an Arrehenius function was used (Badger and Collatz 1977), Parameter (TºC) = Parameter (25ºC) $Q_{10}^{[(25\text{-}T^{\circ}C)/10]}$, in which Q_{10} is the temperature coefficient that represents the factor by which the rate of a reaction increases for every 10ºC rise in the temperature. The tabled values of K_c and K_o at 25ºC (650 μbar and 450 mbar, respectively) and the Q_{10} values for K_c and K_o (2.24 and 1.63, respectively) (von Caemmerer 2000) were used to calculate the K_c and K_o at 20ºC. The K_p value tabled at 25ºC (80 μbar) (von Caemmerer 2000) and the Q_{10} value for K_p of 1.603, calculated from the PEPC activation energy value (34.8 KJ mol^{-1}) obtained for *P. dilatatum* plants at temperatures higher than 20ºC (Cavaco 2000), were

used to calculate the K_p value at 20ºC. The parameters and the values used to apply the photosynthetic model are described in Table 6.1..

Table 6.1. Parameters used (corrected to 20ºC) for the application of the C_4 photosynthetic model based on the CO_2-response curves (von Caemmerer 2000).

Parameter	Value	Description
K_c	0.973 mbar	Michaelis-Menten constant of Rubisco for CO_2 (variable with temperature, corrected to 20ºC)
K_o	575 mbar	Michaelis-Menten constant of Rubisco for O_2 (variable with temperature, corrected to 20ºC)
K_p	101 μmol mol^{-1}	Michaelis-Menten constant of PEPC for CO_2 (variable with temperature, corrected to 20ºC)
O	21%	O_2 partial pressure in the bundle sheath and mesophyll cells
g_{bs}	3 mmol m^{-2} s^{-1}	Bundle-sheath conductance to CO_2
g_i	2 mol m^{-2} s^{-1}	Mesophyll conductance to CO_2
R_d	0.01*Vcmax	Mitochondrial respiration
R_m	0.5*R_d	Mesophyll mitochondrial respiration
γ^*	0.000193	Half the reciprocal of Rubisco speficity
x	0.4	Partitioning factor of electron transport rate
θ	0.7	Empirical curvature factor
f	0.15	Factor that corrects for spectral quality of light
abs	0.85	Absorptance of leaves
I	1000 μmol m^{-2} s^{-1}	Irradiance used
Ia	361.2 μmol m^{-2} s^{-1}	Total absorbed irradiance

A similar approach to that described by Massad *et al.* (2007) was used to estimate *Vpmax*, *Vcmax* and *Jmax*. Firstly, an asymptotic exponential model was chosen as the one that provided the best description of the variation of *A* with intercellular CO_2 concentration (*Ci*) for each individual plant according to the least square method using the program Statistical Package for Social Sciences (SPSS) (version 15.0, 2006, SPSS Inc., Chicago, Illinois, USA). The asymptotic exponential model was used to obtain values of *A* for any value of *Ci* at chosen intervals. Both the enzyme-limited

photosynthesis equations and the light- and electron-transported-limited photosynthesis equations, described in Table 6.2., were firstly applied to each individual plant curve for the range of *Ci* obtained (near zero to near 500 μL L^{-1}) using a step-size of 5 μL L^{-1} *Ci*, allowing the estimation of *Vpmax*, *Vcmax* and *Jmax* values for each *Ci*. The estimated values of *Vpmax*, *Vcmax* and *Jmax* were plotted against *Ci* and the mean values for each individual plant curve were found from 50 to 80 μL L^{-1} *Ci* for *Vpmax* and from 300 to 500 μL L^{-1} *Ci* for *Vcmax* and *Jmax*. Then, the mean values for each individual plant CO_2-response curves were used to found the mean values of *Vpmax*, *Vcmax* and *Jmax* for each treatment (control, one and two nigh-chilling), either with light orientated to the adaxial or abaxial leaf surface.

Both the enzyme-limited photosynthesis equations and the light- and electron-transported-limited photosynthesis equations were solved using the program Mathematica (version 6.0, 2007, Wolfram Research Inc., Illinois, USA). The estimation of the *Vpmax* and *Vcmax* values for each *Ci* required two groups of *A* and *Ci* values. Each group of *A* and *Ci* (differencing in 5 μL L^{-1} *Ci*) uses the three equations of enzyme-limited photosynthesis (Table 6.2.), and thus the estimation of the *Vpmax* and *Vcmax* values for each *Ci* required the solving of a six equations system. The estimation of *Vpmax* and *Vcmax* also allowed the estimation of mitochondrial respiration and mesophyll mitochondrial respiration (respectively, R_d = 0.01 *Vcmax* and $R_m = 0.5R_d$ accordingly to von Caemmerer 2000) for each *Ci*, which were used in the estimation of *Jmax*. The estimation of the *Jmax* value for each *Ci* just required one group of *A* and *Ci* values, using a system of three equations of the light- and electron-transport limited photosynthesis (Table 6.2.). The solving of the system allowed the estimation of the predicted electron transport rate (*Jt*) for each *Ci*. Then, the *Jmax* value for each *Ci* was estimated through the application of the most frequently used equation to characterize the relationship between the electron transport rate and the absorbed irradiance (Table 6.2.).

6.3.4.2. Light-response curves

The whole leaf photosynthetic light-response curves were analysed regarding the apparent quantum yield (ϕ), curvature degree (θ), maximal rate of photosynthesis (*Amax*) and mitochondrial respiration (*Rd*), accordingly to von Caemmerer (2000) and

Lambers *et al.* (1998), through the program Statistica 8.0, 2007 (StatSoft, Inc., OK, USA).

Table 6.2. Equations for enzyme-limited photosynthesis and for light- and electron-transport-limited photosynthesis used to predict the maximal phospho*enol*pyruvate carboxylase (PEPC) carboxylation rate (*Vpmax*), maximal ribulose-1,5-bisphosphate (Rubisco) carboxylation rate (*Vcmax*) and maximal electron transport rate (*Jmax*) from the CO_2-response curves of the whole leaf photosynthesis accordingly to von Caemmerer (2000). See Table 6.1. for all abbreviations, but *A* (net CO_2 assimilation rate), *Ci* (intercellular CO_2 concentration), *Cm* (mesophyll cells CO_2 concentration), *Cs* (bundle sheath cells CO_2 concentration) and *Jt* (predicted electron transport rate).

Parameter	Equations
Vpmax and Vcmax	Equations for enzyme-limited photosynthesis: $A = gi \times (Ci - Cm)$ $A = \frac{Cs \times Vc\max}{Cs + Kc \times \left(1 + O/Ko\right)}\left(1 - \frac{\gamma^* \times O}{Cs}\right) - Rd$ $A = \frac{Cm \times Vp\max}{Cm + Kp} - gbs \times (Cs - Cm) - Rm$
Jmax	Equations for light- and electron-transported-limited photosynthesis: $A = gi \times (Ci - Cm)$ $A = \frac{\left(1 - \gamma^* \times O/Cs\right) \times (1 - x) \times Jt}{3 \times \left(1 + {}^{7} \times \gamma^* \times O/Cs\right)} - Rd$ $A = \frac{x \times Jt}{2} - gbs \times (Cs - Cm) - Rm$
	Equation characterizing the relationship between the electron transport rate and the absorbed irradiance: $Ia = \frac{I \times abs \times (1 - f)}{2}$ $Jt = \frac{Ia + J\max - \sqrt{(Ia + J\max)^2 - 4 \times \theta \times Ia \times J\max}}{2 \times \theta}$

The equation used for the estimation of the light-response curve parameters is described in Chapter 3, Table 3.1.. The C_4 photosynthetic light-response curve model requires that the CO_2 concentration at which the curve was performed is saturating. Since the light-response curve was performed at 360 μL L^{-1} CO_2 (corresponding to a *Ci* value around 120-200 μL L^{-1}), a saturating CO_2 concentration accordingly to Fig. 6.1., the predicted ϕ, θ, *Amax* and *Rd* values from the application of the model can be considered as real.

Each parameter was calculated for each individual plant curve and then its mean value was obtained for each dark-chilling and light orientation treatment.

6.3.5. Statistical analysis

The data were statistically analysed using parametric tests at a stringency of $P<0.05$. The significance of variation in mean values for pigment, protein, and whole leaf photosynthetic model determinations was analysed using an ANOVA followed by a Tukey HSD test. All the analyses were performed separately for each parameter.

6.4. Results

6.4.1. Leaf pigment and protein contents after a dark-chilling stress

Plants subjected to dark-chilling show a decrease in the content of both chl*a* and chl*b* in the first hours of the following day (Table 6.3.). The increase in the ratio chl*a*/chl*b* indicates that the decrease in chl*b* (19%) was higher than the decrease in chl*a* (9%) in one night-chilled plants. On the contrary, the decrease of chl*a* and chl*b* was similar in two nights-chilled plants (respectively 13% and 14%) and, thus, the ratio chl*a*/chl*b* was not significantly different from the one obtained for the control plants. Carotenoids content just decreased significantly after the plants were subjected to the second consecutive night of chilling (Table 6.3.). The Chl *a*+*b*/carotenoids ratio was not affected by the dark-chilling. Soluble protein content was not affected by either one or two nights of chilling (Table 6.3.).

Table 6.3. Leaf pigment and protein content in control, one and two nights-chilled *Paspalum dilatatum* plants. Data represent the average ± SE of seven leaves *per* treatment. Statistical analysis was performed separately for each parameter. The different letters represent statistical differences at P<0.05. Chl, chlorophyll.

	Control	1 Night Chilling	2 Nights Chilling
Chl*a* (mg m^{-2})	572 ± 15.7 a	518 ± 14.7 b	499 ± 4.9 b
Chl*b* (mg m^{-2})	156 ± 4.9 a	127 ± 4.3 b	135 ± 2.0 b
Chl*a*+*b* (mg m^{-2})	727 ± 2.0 a	645 ± 18.9 b	634 ± 5.7 b
Carotenoids (mg m^{-2})	114 ± 3.3 a	108 ± 2.6 ba	100 ± 1.4 b
Chl*a*/Chl *b* ratio	3.7 ± 0.06 a	4.1 ± 0.04 b	3.7 ± 0.05 ab
Chl*a*+*b*/Carotenoids ratio	6.4 ± 0.07 ab	6.0 ± 0.06 a	6.3 ± 0.10 ab
Soluble proteins (mg m^{-2})	3084 ± 148.9 a	2773 ± 65.3 a	2797 ± 78.8 a

6.4.2. Dark-chilling effects on the whole leaf photosynthesis and stomatal conductance

6.4.2.1. CO_2-response curves

Whole leaf *A* increased with *Ci* in all treatments regardless of the light orientation (Fig. 6.1.*a*,*b*). However, steady-state rates of photosynthesis in the control and in two nights-chilled plants were slightly higher when light was oriented to the adaxial surface (Fig. 6.1.*a*,*b*). On the contrary, the steady-state rate of photosynthesis was similar after one night-chilling either with light oriented to the adaxial or the abaxial leaf surface.

Different responses of chilled plants were found under adaxial and abaxial illumination of leaves. Adaxial illumination resulted in a decrease of the steady-state rates of photosynthesis in one night-chilled plants (Fig. 6.1.*a*). Two night-chilled plants showed an intermediate value for the steady-state rates of photosynthesis between the control and one-night chilled plants (Fig. 6.1.*a*). On the contrary, abaxial illumination of leaves resulted in a slightly higher decrease of the steady-state rates of photosynthesis after two nights of chilling (Fig. 6.1.*b*). One night-chilled plants showed a steady-state rate of photosynthesis between that of control and two nights-chilling (Fig. 6.1.*b*). The initial slope of the CO_2-response curve was not very different between the control and

the stressed plants either under adaxial or abaxial illumination, although it seems slightly lower in dark-chilled plants under adaxial illumination.

Whole leaf *gs* decreased with increasing *Ci* and showed similar trends whether light was supplied via the adaxial or abaxial surface of the leaves (Fig. 6.1.*c*,*d*). However, the overall values were slightly lower under adaxial illumination. Also under this illumination chilled plants showed slightly higher *gs* values at elevated *Ci* (Fig. 6.1.*c*), whereas under abaxial illumination similar *gs* values were obtained for control and chilled plants (Fig. 6.1.*d*).

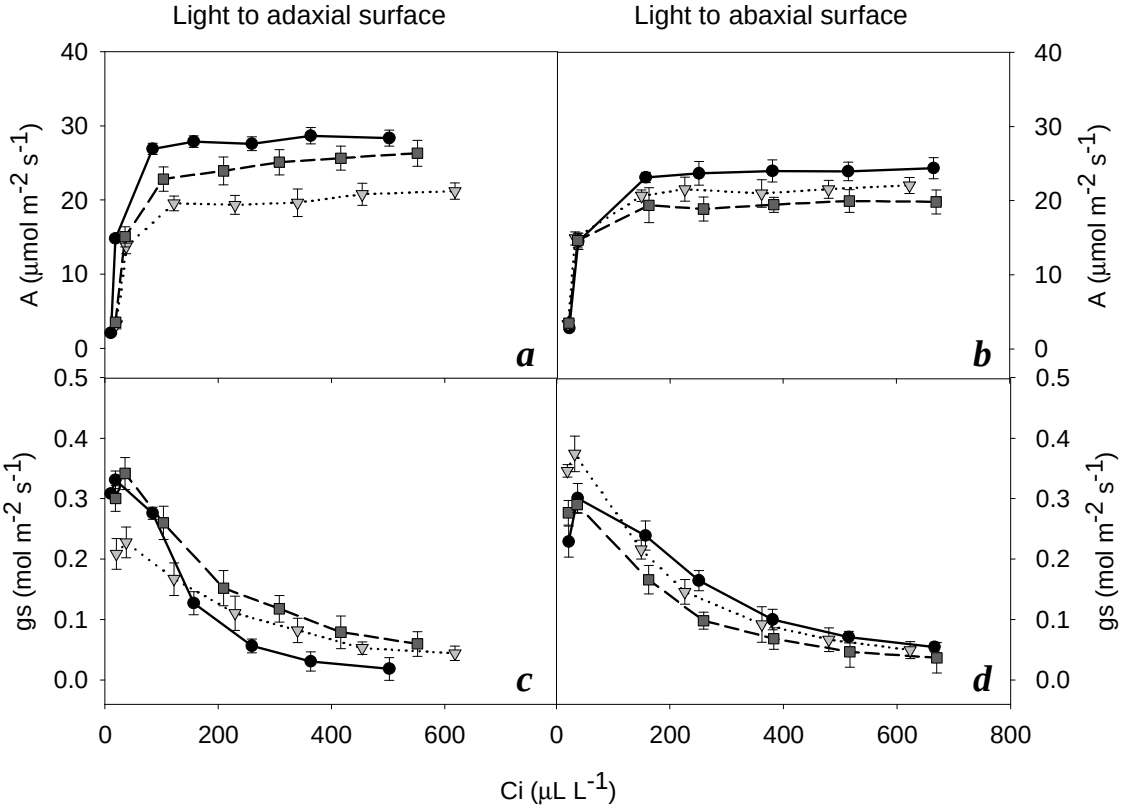

Figure 6.1. The effect of light orientation on whole leaf CO_2-response curves for net CO_2 assimilation rate (*A*, ***a***, ***b***) and stomatal conductance to water vapour (*gs*, ***c***, ***d***) in control (black circles), one (light grey inverted triangles) and two (dark grey squares) nights-chilled *Paspalum dilatatum* plants. The light source was oriented either to the adaxial surface (***a***, ***c***) or to the abaxial surface (***b***, ***d***). Data are the mean values ± SE of three plants at each CO_2 concentration. Ci, intercellular CO_2 concentration.

6.4.2.2. Light-response curves

The whole leaf light-response curves for photosynthesis were similar regardless of leaf orientation towards light and dark-chilling treatment (Fig. 6.2.*a*,*b*). Independently

of the leaf surface that was illuminated, the whole leaf *gs* increased with increasing irradiance (Fig. 6.2.*c*,*d*). Control and two night-chilled plants showed similar *gs* values, while one night-chilled plants tend to show slightly higher values, regardless of the leaf orientation towards light (Fig. 6.2.*c*,*d*).

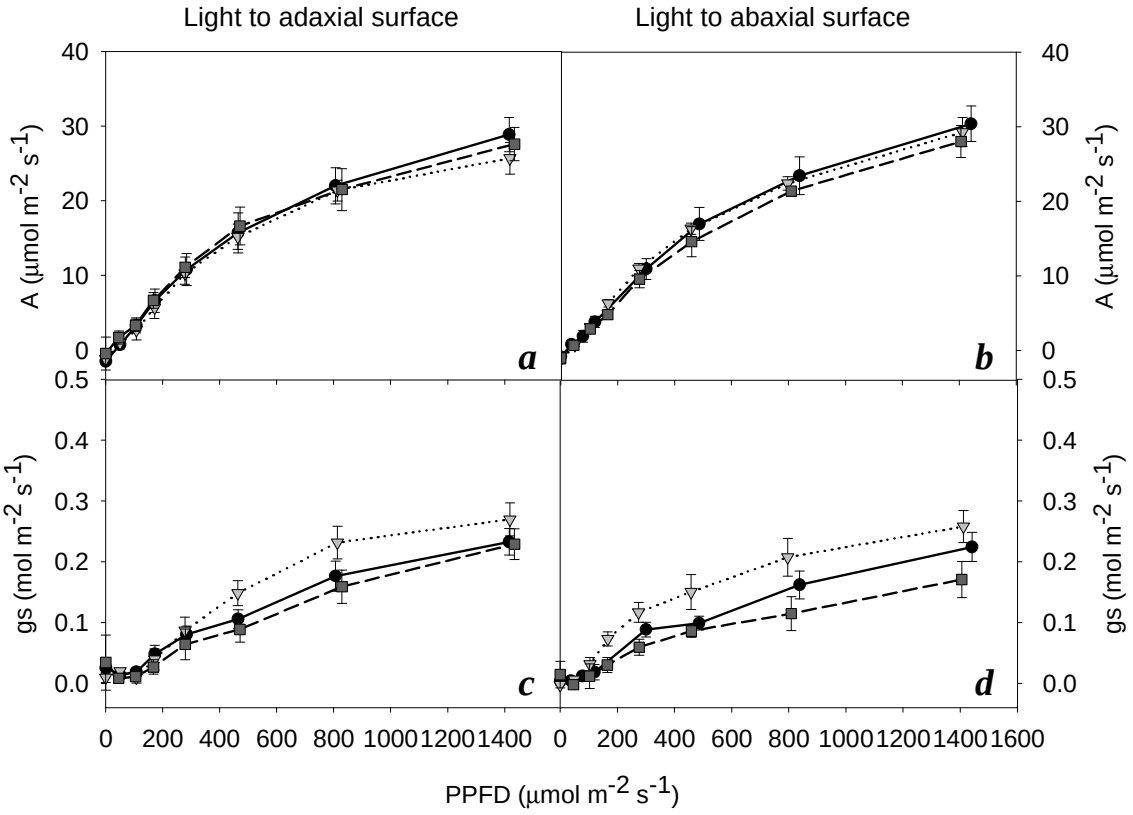

Figure 6.2. The effect of light orientation on whole leaf light-response curves for net CO_2 assimilation rate (*A*, ***a***, ***b***) and stomatal conductance to water vapour (*gs*, ***c***, ***d***) in control (black circles), one (light grey inverted triangles) and two (dark grey squares) nights-chilled *Paspalum dilatatum* plants. The light source was oriented either to the adaxial surface (***a***, ***c***) or to the abaxial surface (***b***, ***d***). Data are the mean values ± SE of three plants at each light intensity. PPFD, photosynthetic photon flux density. The PPFD values correspond to leaf incident light intensity.

6.4.3. Dark-chilling effects on the adaxial and abaxial photosynthesis and stomatal conductance

6.4.3.1. CO_2-response curves

Under adaxial illumination, the adaxial surface of the control plants seems to have slightly lower values of *A* at low *Ci* in relation to the abaxial surface (Fig. 6.3.*a*,*b*).

However, steady-state rates of photosynthesis in the control plants were slightly higher on the adaxial surface. One night-chilled plants tend to show lower steady-state rates of photosynthesis on the adaxial surface when light was directly oriented to this surface (Fig. 6.3.*a*). However, similar steady-state rates of photosynthesis were found in the control and two nights-chilled plants. On the contrary, the steady-state rates of photosynthesis on the abaxial surface were not affected by dark-chilling (Fig. 6.3.*b*).

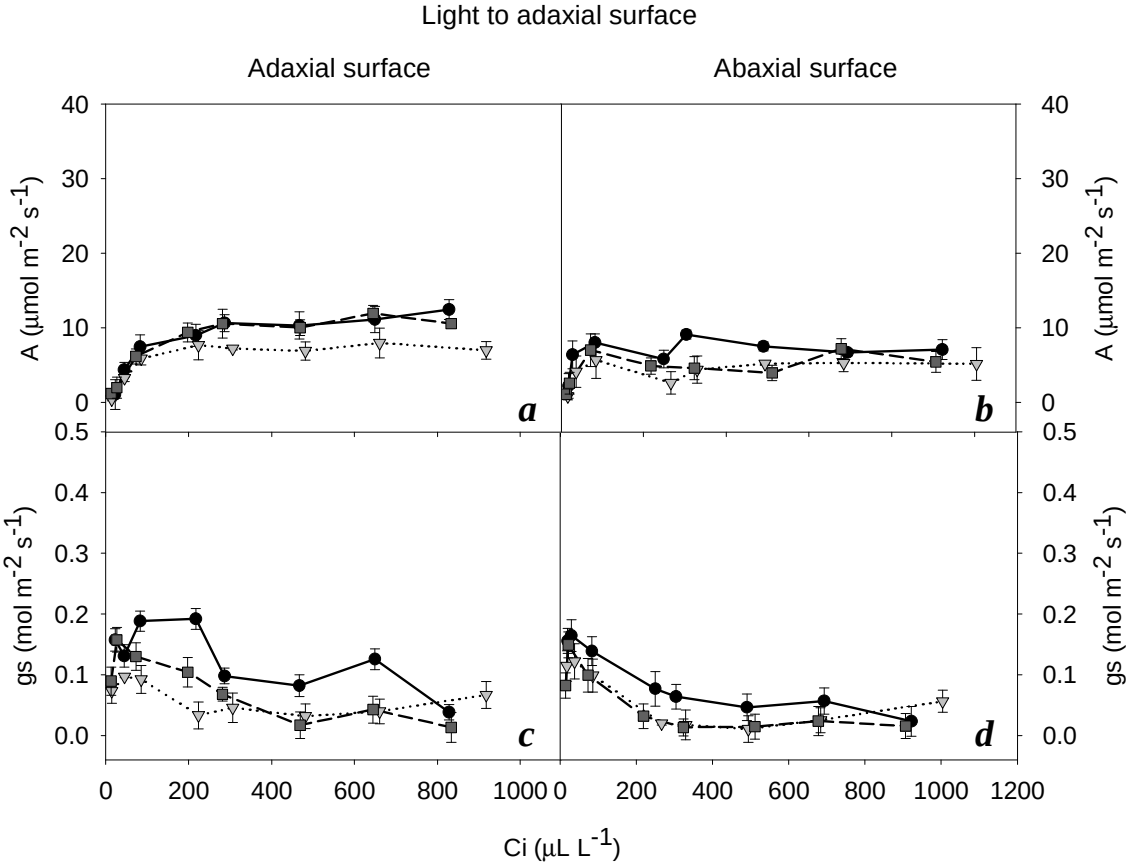

Figure 6.3. The CO_2-response curves for net CO_2 assimilation rate (*A*, ***a***, ***b***) and stomatal conductance to water vapour (*gs*, ***c***, ***d***) on the adaxial (***a***, ***c***) and abaxial (***b***, ***d***) surfaces in control (black circles), one (light grey inverted triangles) and two (dark grey squares) nights-chilled *Paspalum dilatatum* plants. The light source was oriented to the adaxial surface. Data are the mean values ± SE of three plants from each CO_2 concentration. Ci, intercellular CO_2 concentration.

In contrast to what was observed under adaxial illumination, when light was oriented directly to the abaxial surface the steady-state rates of photosynthesis in the control plants were higher on the abaxial surface and almost completely null on the adaxial surface (Fig. 6.4.*a*,*b*). No difference on the CO_2-response curves was found

between the control and dark-chilled plants, either on the adaxial or on the abaxial leaf surfaces.

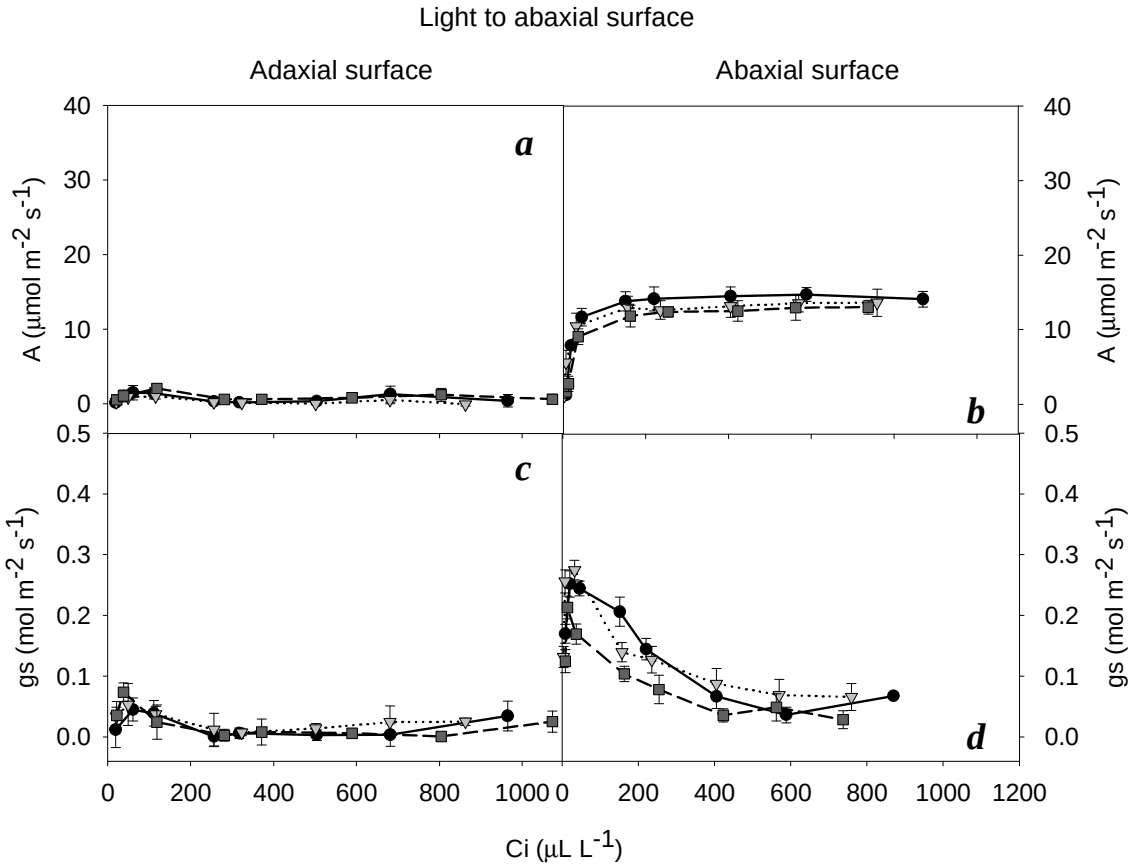

Figure 6.4. The CO_2-response curves for net CO_2 assimilation rate (*A*, ***a***, ***b***) and stomatal conductance to water vapour (*gs*, ***c***, ***d***) on the adaxial (***a***, ***c***) and abaxial (***b***, ***d***) surfaces in control (black circles), one (light grey inverted triangles) and two (dark grey squares) nights-chilled *Paspalum dilatatum* plants. The light source was oriented to the abaxial surface. Data are the mean values ± SE of three plants from each CO_2 concentration. Ci, intercellular CO_2 concentration.

The *gs* values decreased with increasing *Ci* in both leaf surfaces either under adaxial (Fig. 6.3.*c*,*d*) or abaxial (Fig. 6.4.*c*,*d*) illumination. However, while under adaxial illumination both the adaxial and the abaxial surfaces presented open stomata, under abaxial illumination only the stomata on the abaxial surface were open. Furthermore, the abaxial illumination of the leaves resulted in higher *gs* values on the abaxial surface both in control and dark-chilled plants (Fig. 6.4.*d*) than the ones observed under adaxial illumination (Fig. 6.3.*d*). Under adaxial illumination the dark-chilling stress tend to slightly decrease *gs* similarly on both leaf sides (Fig. 6.3.*c*,*d*). However, under abaxial illumination *gs* on the abaxial surface of dark-chilled plants

seem to be slightly lower at low *Ci* but similar to the control values at high *Ci* (Fig. 6.4.*d*). One and two nights-chilled plants generally presented similar *gs* values.

6.4.3.2. Light-response curves

The light-response curve for photosynthesis on each leaf surface increased with irradiance in a similar manner when light was oriented to the adaxial surface (Fig. 6.5.*a*,*b*). When light was oriented to the abaxial side, *A* values on the adaxial surface remained close to the compensation point regardless of the light intensity applied (Fig. 6.6.*a*). In contrast, the abaxial surface showed even higher *A* values than when light was oriented to the adaxial surface (Fig. 6.5.*b*, Fig 6.6.*b*). No difference was observed among control, one and two night-chilled plants under adaxial or abaxial illumination (Fig. 6.5.*a*,*b*, Fig. 6.6.*a*,*b*).

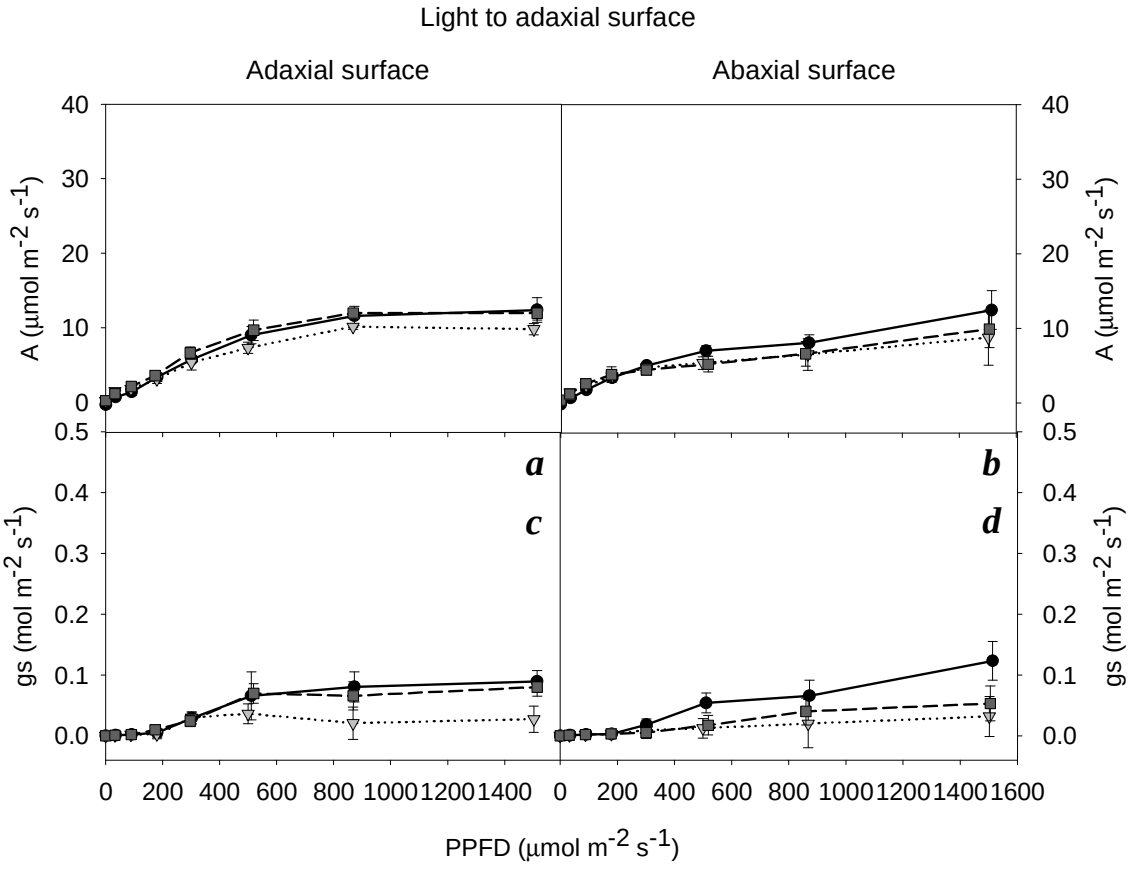

Figure 6.5. The light-response curves for net CO_2 assimilation rate (*A*, ***a***, ***b***) and stomatal conductance to water vapour (*gs*, ***c***, ***d***) on the adaxial (***a***, ***c***) and abaxial (***b***, ***d***) surfaces in control (black circles), one (light grey inverted triangles) and two (dark grey squares) nights-chilled *Paspalum dilatatum* plants. The light source was oriented to the adaxial surface. Data are the mean values ± SE of three plants from each light intensity. PPFD, photosynthetic photon flux density. The PPFD values correspond to leaf incident light intensity.

When light was oriented to the adaxial leaf surface *gs* values of the control and dark-chilled plants increased with light intensity and were similar on both leaf surfaces (Fig. 6.5.*c*,*d*). There is, however, a slight tendency for a higher *gs* in control than in one night-chilled plants. On the contrary, under abaxial illumination higher values of *gs* were found on the abaxial surface, while almost null values were observed on the adaxial side (Fig. 6.6.*c*,*d*). The stomatal conductance on each leaf surface after dark-chilling seems to be less affected under this light orientation.

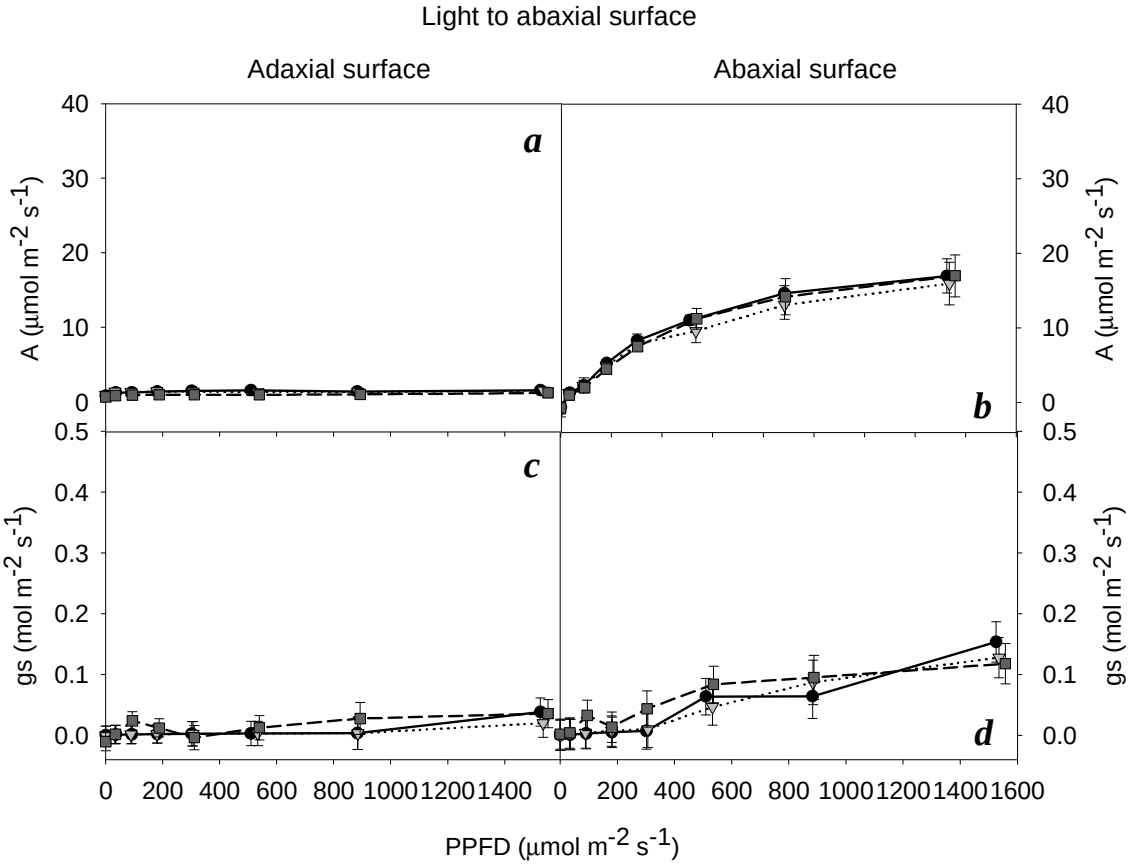

Figure 6.6. The light-response curves for net CO_2 assimilation rate (*A*, ***a***, ***b***) and stomatal conductance to water vapour (*gs*, ***c***, ***d***) on the adaxial (***a***, ***c***) and abaxial (***b***, ***d***) surfaces in control (black circles), one (light grey inverted triangles) and two (dark grey squares) nights-chilled *Paspalum dilatatum* plants. The light source was oriented to the abaxial surface. Data are the mean values ± SE of three plants from each light intensity. PPFD, photosynthetic photon flux density. The PPFD values correspond to leaf incident light intensity.

6.4.4. Dark-chilling effects on the estimated parameters from the whole leaf photosynthetic models

Leaf orientation to light has affected the estimated whole leaf *Vpmax*, *Vcmax* and *Jmax* values obtained from control plants, decreasing respectively by 48%, 19% and 22% under abaxial illumination (Table 6.4.). The estimated values for the whole leaf *Amax*, ϕ, θ and *Rd* were not affected by light orientation in control plants (Table 6.5.).

Table 6.4. Whole leaf maximal phospho*enol*pyruvate carboxylase (PEPC) carboxylation rate (*Vpmax*), maximal ribulose-1,5-bisphosphate (Rubisco) carboxylation rate (*Vcmax*) and maximal electron transport rate (*Jmax*) in control, one and two nights-chilled *Paspalum dilatatum* plants. Data represent the average ± SE of three plants *per* treatment and were calculated following the application of the C_4 photosynthetic model of von Caemmerer (2000) to the whole leaf CO_2-response curves (See Fig. 6.1.*a,b*). Statistical analysis was performed separately for each parameter. The different letters represent statistical differences at P<0.05.

	Vpmax (μmol m^{-2} s^{-1})	Vcmax (μmol m^{-2} s^{-1})	Jmax (μmol m^{-2} s^{-1})
Light Adaxial			
Control	111 ± 4.3 a	32 ± 1.0 a	214 ± 15.6 a
1 Night Chilling	52 ± 1.4 c	22 ± 0.7 c	144 ± 11.3 b
2 Nights Chilling	63 ± 1.0 bc	28 ± 1.1 ab	183 ± 10.2 ab
Light Abaxial			
Control	58 ± 2.9 bc	26 ± 0.5 b	167 ± 1.6 b
1 Night Chilling	67 ± 4.8 b	24 ± 0.8 bc	152 ± 6.4 bc
2 Nights Chilling	66 ± 2.5 bc	22 ± 1.1 c	148 ± 3.5 c

One night-chilling decreased the *Vpmax*, *Vcmax*, *Jmax* and *Amax* values by 30 to 50% when light was oriented to the adaxial leaf surface (Table 6.4., Table 6.5.). However, in two night-chilled plants these parameters showed similar values to that of control, except for *Vpmax* that presented a similar decrease to that found after one night-chilling (around 45%) (Table 6.4.). When light was oriented to the abaxial leaf surface, no changes were found in *Vpmax* and *Amax* between control and dark-chilled plants

(Table 6.4., Table 6.5.). Estimated values for *Vcmax* and *Jmax* decreased only after two nights of chilling by 15% and 11%, respectively, in relation to control values.

The ϕ, θ and *Rd* did not change either with dark-chilling or with light orientation towards the leaf (Table 6.5.).

Table 6.5. Whole leaf maximal rate of photosynthesis (*Amax*), apparent quantum yield (ϕ), curvature degree (θ) and mitochondrial respiration (*Rd*) in control, one and two nights-chilled *Paspalum dilatatum* plants. Data represent the average ± SE of three plants *per* treatment and was calculated accordingly to von Caemmerer (2000) and Lambers *et al.* (1998) from the whole leaf light-response curves (See Fig. 6.2.*a,b*). Statistical analysis was performed separately for each parameter. The different letters represent statistical differences at P<0.05.

	Amax (μmol m^{-2} s^{-1})	ϕ ($*10^2$ μmol μmol^{-1})	θ (relative units)	Rd (μmol m^{-2} s^{-1})
Light Adaxial				
Control	41 ± 2.4 a	5.7 ± 0.58 a	0.64 ± 0.045 a	1.9 ± 0.06 a
1 Night Chilling	26 ± 0.6 b	4.6 ± 0.66 a	0.82 ± 0.044 a	1.8 ± 0.08 a
2 Nights Chilling	39 ± 2.7 a	6.5 ± 0.23 a	0.69 ± 0.090 a	1.9 ± 0.13 a
Light Abaxial				
Control	43 ± 1.7 a	4.2 ± 0.40 a	0.64 ± 0.073 a	1.6 ± 0.05 a
1 Night Chilling	37 ± 1.0 a	5.4 ± 0.12 a	0.61 ± 0.035 a	1.6 ± 0.03 a
2 Nights Chilling	37 ± 1.0 a	4.6 ± 0.50 a	0.64 ± 0.073 a	1.7 ± 0.07 a

6.5. Discussion

6.5.1. Dark-chilling decreased chlorophyll and carotenoids content

In higher plants, Chl*a*, Chl*b* and carotenoids are associated with proteins constituting the pigment-protein complexes that are part of the PSI and PSII structures, which are responsible for the capture and conversion of light into chemical energy (for review see Green and Durnford 1996 and Horton *et al.* 1996). Most Chl*a* and Chl*b* molecules in higher plants are included in the antenna, and only few Chl*a* molecules form the reaction centres which transfer electrons to acceptor molecules.

One or two nights-chilled *P. dilatatum* plants showed a similar decrease in the total leaf chlorophyll content (Table 6.3.). This result contradicts the one obtained by Garstka *et al.* (2007) in which no variation was observed in the total leaf chlorophyll content after transference of dark-chilled pea and tomato plants to light for 3 h. These different results may be related with the sensitivity of the species to cold, as well as with the light intensity used after the dark-chilling treatment, 200 μmol m^{-2} s^{-1} in the study performed by Garstka *et al.* (2007) and 600-650 μmol m^{-2} s^{-1} in this study. The higher light intensity may have increased the susceptibility of chlorophylls to chilling-induced damaged. However, our results confirm the previously reported data obtained for *P. dilatatum* plants under a short- or a long-term chilling regime (Slack *et al.* 1974, Forde *et al.* 1975, Cavaco *et al.* 2003), and for other C_4 crops such as maize and *Sorghum bicolour* (Slack *et al.* 1974, Haldimann *et al.* 1995, Nie *et al.* 1995, Haldimann 1996). On the contrary, in C_3 species with a higher potential to tolerate chilling, as winter cultivars of wheat and rye, spinach and *Arabidopsis thaliana*, either the maintenance of total leaf chlorophyll content or its increase have been reported under long-term chilling (Huner *et al.* 1989, Gray *et al.* 1996, Wu *et al.* 1997). It is widely accepted that the decline in the leaf chlorophyll content is a general symptom of chilling injury, depending on the species sensitivity to chilling, as well as on the type and duration of the stress imposed.

The decrease in the total leaf chlorophyll content observed under cold conditions (Table 6.3.) has been attributed to impaired chlorophyll synthesis and/or to the photo-oxidation of newly synthesized chlorophyll prior to its photo-stable integration in the thylakoid (Hodgins and van Huystee 1985, van Huystee and Hodgins 1989). The lower content of total leaf chlorophyll after the short-term dark-chilling stress could suggest a decreased/limited capacity for light absorption. In fact the activity of PSII is decreased after a dark-chilling stress in soybean and grapevine leaves (van Heerden *et al.* 2003*b*, Bertamini *et al.* 2005, Strauss *et al.* 2007) and in *P. dilatatum* plants under moderate and high light intensities (see Chapter 3). Our results also show that one night-chilling led to a higher decrease of Chl*b* than Chl*a*, a trend that was not observed after two nights-chilling. However, Cavaco *et al.* (2003) showed that in cold-acclimated *P. dilatatum* plants Chl*a* content had a higher contribution for the decrease of total leaf chlorophyll. These opposite results obtained with the same species suggest that the response of pigments content to chilling depends on the type and duration of the stress.

Dark-chilling tended to decrease the carotenoids content in a similar way as the leaf total chlorophyll. Cavaco *et al.* (2003) also found that *P. dilatatum* cold-acclimated plants presented a lower content of carotenoids, but the Chl *a*+*b*/carotenoids ratio decreased, indicating a higher decrease in the total chlorophylls content. Changes in the content and composition of carotenoids are particularly important in the process of cold-acclimation, especially when this is associated to high light intensity. Carotenoids have the dual function as accessory pigments in energy capture and as photosystem photoprotection in the chloroplasts. The latter is achieved through the dissipation of the excessive energy and by scavenging the oxygen radicals generated under photoinhibitory conditions through the mechanism of epoxidation (Bilger and Björkman 1991, Chaumont *et al.* 1995, Verhoeven *et al.* 1996). The photoprotective mechanism against photoinhibition and recovery of the PSII efficiency from chilling stress has been shown to be associated to changes in content and composition of the carotenoids of the xanthophylls cycle (e.g. Haldimann *et al.* 1995, Haldimann 1996, Verhoeven *et al.* 1996, Wang *et al.* 2008).

6.5.2. Soluble protein content was not affected by dark-chilling

Soluble protein content was not affected after one or two nights of chilling in the semi-tolerant species *P. dilatatum* (Table 6.3.), whereas under cold acclimation an increase was observed (Cavaco *et al.* 2003). However, protein synthesis and turnover, and thus its content, are known to decrease in chilling sensitive plants (Lyons 1973), as in soybean plants subjected to three consecutive nights of cold (Strauss *et al.* 2007) and in maize plants under cold acclimation (Naidu *et al.* 2003). On the contrary, the soluble protein content increased in the cold-tolerant *Miscanthus* species under cold acclimation (Naidu *et al.* 2003). The response of protein content to a chilling stress depends on the cold sensitivity of the species studied and on the type and duration of the stress, as already observed for the pigment content.

6.5.3. Dark-chilling effects on whole leaf photosynthesis are more evident when light enters the leaf via the adaxial surface

Dark-chilling is known to decrease photosynthesis under adaxial illumination in several plants, although this decrease depends on the number of cold nights and on the species sensitivity (e.g. van Heerden *et al.* 2003*a*, Feng and Cao 2005, Ludlow and Wilson 1971, Ivory and Whiteman 1978). Under this illumination, the steady-state rate of photosynthesis in the CO_2-response curves decreased more after one than after two nights of chilling (Fig. 6.1.). Adaxial illumination decreased more *Vpmax* than *Vcmax* and *Jmax* after one night-chilling, suggesting that under this light orientation towards the leaf the maximal activity of the first carboxylation enzyme of C_4 plants may be more susceptible to one night-chilling (Table 6.4.). Furthermore, photosynthesis recovered almost completely after the second night of stress, as also shown by the estimated values for *Vcmax* and *Jmax* (Table 6.4.), contrary to the observed for the majority of the sensitive C_3 species (van Heerden *et al.* 2003*a*, Feng and Cao 2005). However, *Vpmax* did not recover after two nights of chilling under adaxial illumination, once more supporting the idea that PEPC carboxylation rate may have a higher sensitivity to dark-chilling.

As observed before in Chapter 5, abaxial illumination of control leaves resulted in a lower whole leaf steady-state photosynthesis rate in the CO_2-response curve (Fig. 6.1.), which may be related with the null photosynthesis measured on the adaxial surface (see Chapter 5 and Fig. 6.4.). In accordance, the predicted *Vcmax* and *Jmax* values also decreased in control plants under abaxial illumination (Table 6.4.). In addition, *Vpmax* also showed a lower value under abaxial illumination in control plants (Table 6.4.).

The effect of dark-chilling on photosynthesis measured when light entered the leaf via the abaxial surface has never been studied. Whole leaf photosynthesis under abaxial illumination was less affected by dark-chilling than under adaxial illumination, just decreasing slightly the steady-state rate of photosynthesis after two nights of chilling, an observation supported by the decrease of *Vcmax* and *Jmax* (Table 6.4.). The non variation of *Vpmax* after dark-chilling (Table 6.4.) suggest that under abaxial illumination the maximal activity of the first carboxylation enzyme of C_4 plants may be less sensitive to dark-chilling, contrary to the observed under adaxial illumination. In

accordance with our results, a study performed with *P. dilatatum* plants grown under CO_2 enrichment showed that adaxial illumination of the leaf resulted in a lower whole leaf photosynthesis, while similar rates of photosynthesis in control and high-CO_2 grown plants were found when light entered the leaf via the abaxial surface (Soares *et al.* 2008). The above data shows that both the dark-chilling and the CO_2 enrichment affected more the photosynthesis measured on the adaxial and abaxial surfaces (with adaxial illumination) than measured just on the abaxial side (with abaxial illumination) in *P. dilatatum* plants, indicating that probably the adaxial leaf side may be more stress sensitive than the abaxial surface in this C_4 monocotyledonous species.

Stomatal limitations to photosynthesis under adaxial illumination are known to occur in dark-chilled plants (Pasternak and Wilson 1972, Ivory and Whiteman 1978, Bauer *et al.* 1985, van Heerden *et al.* 2003*a*). The present results show that at low *Ci* concentrations (in which is included the *Ci* concentration resulted from an atmospheric concentration of 360 $\mu L\ L^{-1}$) *gs* may be limiting the whole leaf photosynthesis in one night-chilled plants, but at elevated *Ci* concentrations the dark-chilling plants showed slightly higher *gs* values (Fig. 6.1.). However, this higher *gs* did not allow these plants to present higher *A* values, suggesting that metabolic restrictions to photosynthesis may have occurred after dark-chilling at higher *Ci* concentrations. It is known that dark-chilling stress restrict the activity of several photosynthetic enzymes both in C_3 and C_4 plants (e.g. Jones *et al.* 1998, Pittermann and Sage 2001, van Heerden *et al.* 2003*a*) and decreased the activity of electron transport chain in C_3 plants (e.g. Bertamini *et al.* 2005, Strauss *et al.* 2007).

The photosynthetic light-response curve and the predicted parameters from this curve show that dark-chilling in general did not alter the whole leaf photosynthesis (Fig.6.2. and Table 6.5.), regardless the light orientation towards the leaf. However, the decrease of *Amax* only under adaxial illumination in one night-chilled plants indicates that dark-chilling affects less the photosynthetic metabolism under abaxial illumination, as also observed in the CO_2-response curves. The recovery of *Amax* in two night-chilled plants under adaxial illumination indicates that plants are responding positively to the imposed stress, as also shown in the CO_2-response curves.

6.5.4. Adaxial/abaxial specification of photosynthesis is maintained after dark-chilling

As observed by Driscoll *et al.* (2006) for maize and Soares *et al.* (2008) for *P. dilatatum* plants grown with CO_2 enrichment, the adaxial/abaxial specification of photosynthesis and stomatal conductance in *P. dilatatum* described in Chapter 5 was maintained after dark-chilling, either in the CO_2- or in the light-response curves. While under adaxial illumination both leaf surfaces photosynthetic rate and stomatal conductance were contributing to the whole leaf photosynthesis, under abaxial illumination only the contribution of the abaxial leaf surface processes were found (Fig 6.3. to Fig. 6.6.).

Adaxial illumination of leaves tends to decrease the steady-state rate of photosynthesis on the adaxial surface of one night-chilled plants in the CO_2-response curves, a phenomenon that was not observed on the abaxial surface (Fig 6.3.), suggesting that the abaxial surface may be less sensitive to dark-chilling. Since the steady-state rate of photosynthesis can be limited by *Vcmax* (i.e. Rubisco capacity) and *Jmax* (which is involved in the RuBP regeneration capacity, PEP regeneration and *P*i regeneration capacity) (von Caemmerer and Furbank 1999, von Caemmerer 2000) we may suggest that one or all this factors may be more affected on the adaxial than on the abaxial leaf surface after dark-chilling. The decrease in whole leaf steady-state rate of photosynthesis after one-night chilling under adaxial illumination may be related to a decrease on adaxial surface photosynthesis. The tendency to recover of the adaxial surface photosynthesis to values of the control plants after two nights-chilling is in accordance with the tendency to recover of whole leaf photosynthesis and supports the suggestion that adaxial surface photosynthesis may be more affected by one night-chilling.

As for the whole leaf photosynthesis, dark-chilling did not affect or affected slightly the photosynthesis on both leaf surfaces under abaxial illumination, either in the CO_2- or in the light-response curves (Fig. 6.4., Fig. 6.6.). The slightly or null variation of whole leaf photosynthesis after dark-chilling under abaxial illumination may be related to the fact that the surface more sensitive to dark-chilling, the adaxial surface, presents a null or low value under this illumination, while the surface less sensitive to the stress presents much higher photosynthetic rates.

The same behaviour of photosynthesis on each leaf surface with light orientation towards the leaf has also been observed by Soares *et al.* (2008) with *P. dilatatum* plants grown at elevated CO_2, suggesting that the different response of the adaxial and abaxial leaf surfaces photosynthesis may be a common feature under several stress situations. Once more these results show that the two leaf surfaces in *P. dilatatu*m are not functionally symmetrical, as the leaf structure suggests (Cavaco 2000), and that they respond differently to environmental stresses.

6.5.5. Stomatal conductance on each leaf surface seems to be similarly affected after dark-chilling

Although the dark-chilling has led to slight differences in whole leaf and both leaf surfaces stomatal conductance (Fig. 6.1. to Fig. 6.6.), variations are low and no conclusive suggestion can be drawn. However, is seems that there is a tendency for the stomatal conductance to be lower in dark-chilled plants on both surfaces, especially under adaxial illumination.

Our results contradict those in the literature, where the stomata on the adaxial and abaxial surface photosynthesis have presented different sensitivities to environmental stresses under adaxial leaf illumination (Turner and Singh 1984, Pearson *et al.* 1995, Lu 1988, Wang *et al.* 1998). This different result may be related to type of stress and to the different period of time the plants were stressed, a long period in the mentioned works and a short period in the present assays.

6.6. Conclusion

We conclude that in *P. dilatatum* the dark-chilling decreased more the chlorophyll *b* than chlorophyll *a* and did not affect the soluble protein content. Leaf orientation towards light is crucial for whole leaf photosynthesis in control plants. Two nights of chilling affected less the whole leaf photosynthetic rate than one night-chilling under adaxial illumination. Photosynthetic rate seems to be more affected by dark-chilling under this light illumination than under abaxial illumination, both in the whole leaf and in each leaf surface separately. Although the dorso-ventral surface specificity of

photosynthesis and stomatal conductance is maintained after the dark-chilling, photosynthesis tends to be more affected on the adaxial than on the abaxial leaf surface in contrast with the stomatal conductance that seems not to be differently affected on each leaf surface.

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6.8. References

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Chapter 7.
General discussion and future perspectives.

7. General discussion and future perspectives.

Temperature is one of the most important and fluctuating environmental factors limiting plant distribution and growth. Plants with the C_4 photosynthetic metabolism generally dominate the warm climate regions on Earth, being relatively rare in the cool climates characteristic of high latitudes or high elevations (Hattersley 1983, Taub 2000). Globally, C_4 plants are scarce when average growing season temperatures are less than 16ºC (Sage *et al.* 1999). The expected increase in temperature due to climate changes (IPCC 2007) may enhance the proportion of these plants, which contribute at the moment for 30% of the terrestrial primary productivity (Gillon and Yakir 2001). However, the occurrence of low temperatures at night before a mild day may increase the damages due to cold stress (Bracale and Coraggio 2003), greatly affecting the production of C_4 plants. Thereby, the study of the effects of dark-chilling on subsequent photosynthesis at higher temperatures is of particular interest (Allen and Ort 2001), especially in plants with this metabolism.

Similar to the leaves of other monocotyledonous species, *P. dilatatum* leaves grow vertically from the stem and then bend over so that the adaxial surface tends to receive more light directly than the abaxial surface. However, the leaves may curl as they expand or in response to stress conditions, so that both surfaces of the same leaf can receive light directly. Thus, an accurate knowledge of the photosynthetic responses to leaf orientation in pasture and forage grasses such as *P. dilatatum* is crucial in any prediction of plant behaviour, both under control and stress situations.

7.1. Dark-chilling effects on photosynthesis depend on the number of nights at low temperature

Our results show that the changes caused by a short-term dark-chilling depend on the number of chilling nights and are more pronounced on the first night than on the second consecutive night of chilling, as observed earlier for the majority of C_3 plants (e.g. Bauer *et al.* 1985, van Heerden *et al.* 2003). After the disturbance of one night-

chilling, the C_4 plants studied tend to reach a new homeostatic equilibrium after the second night at low temperature (see Chapter 2 and Chapter 6).

The recovery of photosynthesis and some leaf parameters on the second night of chilling seems to depend on the species studied (see Chapter 2). The recovery of photosynthesis and stomatal conductance was higher in *P. dilatatum* (NADP-ME, NADP-malic enzyme) than in *Z. japonica* (PEPCK, phospho*enol*pyruvate carboxykinase), suggesting that the photosynthetic metabolism in the latter species is more sensitive to dark-chilling. In *C. dactylon* (NAD-ME, NAD-malic enzyme) the dark-chilling did not affect the photosynthetic rate. The specific leaf area (*SLA*) was not affected by dark-chilling in *Z. japonica*, but recovered completely after two nights-chilled plants of *P. dilatatum* and *C. dactylon*. While the dry/fresh weight ratio (*DW/FW ratio*) recovered after the second night of chilling in *C. dactylon*, this recovery was not complete in *P. dilatatum*.

The recovery or tendency to recover of photosynthesis and stomatal conductance after the second night of chilling in relation to control and one night-chilled plants also depends on the light intensity at which plants were grown and at witch the assays were performed (see Chapter 2 and Chapter 6). In Chapter 2 photosynthesis recovered totally to control values after the second night of chilling in *P. dilatatum*, whereas in Chapter 6 this recovery was not fully completed. The higher light intensities used in Chapter 6 for the growth and gas-exchange measurements may have had a deleterious effect on the recovery of photosynthetic rate after the second night-chilling.

7.2. One night-chilling effects on photosynthesis depend on the light intensity on the subsequent warm day

Photosynthetic light-response curves and simultaneous gas-exchange measurements at three increasing light intensities (see Chapter 3 and Chapter 6) show that after one night of chilling the changes in the photosynthetic rate are dependent on the light intensity at which plants were subjected, as also observed by Feng and Cao (2005) in a C_3 species. However, it seems that the response of photosynthesis after the dark-chilling in *P. dilatatum* and *C. dactylon* varies with the velocity of the increase in light intensity (see Chapter 3). In addition, the response of photosynthesis after the dark-

chilling in these two species did not follow the same pattern with the different velocity of light increase. While the decrease on photosynthesis after the dark-chilling occurred at the moderate light intensity in *P. dilatatum* when light was increased suddenly, it just decreased in *C. dactylon* at the higher light intensity when light was gradually increased. The above results indicate that the photosynthetic process of the three C_4 species studied after dark-chilling may present different sensitivity to a sudden or slow increase in light intensity.

Although more evident when the three light intensities were imposed suddenly than slowly, the three C_4 species present a different sensitivity of photosynthesis to light intensity after the dark-chilling stress (see Chapter 2 and Chapter 3). While in all species the low light intensity of 200 μmol m^{-2} s^{-1} did not decrease photosynthesis, moderate and high light intensities (530 and 1300 μmol m^{-2} s^{-1}, respectively) decreased CO_2 assimilation rate only in *P. dilatatum* and *Z. japonica*, although in a slightly higher percentage in the later species. Results suggest that *C. dactylon* was the species with the photosynthetic process less sensitive to the dark-chilling stress, while *Z. japonica* seems to present the higher vulnerability of that process (see Chapter 2 and Chapter 3). Furthermore, results suggest that one night at low temperature altered the capacity of these C_4 plants to face an increase in light in the following warm photoperiod, although less evident in the NAD-ME species *C. dactylon*.

The photosynthetic light-response curves from Chapter 3 show that in *P. dilatatum* one night of chilling started to decrease the photosynthetic rate between a light intensity of 530 to 1300 μmol m^{-2} s^{-1}. However, *P. dilatatum* light-response curve results from Chapter 6 indicates that one night of chilling did not affect the whole leaf photosynthesis under adaxial illumination of the leaf at least up to a light intensity of 1400 μmol m^{-2} s^{-1}. This different result may be explained by the plants growth light conditions (250-300 μmol m^{-2} s^{-1} in Chapter 3 and 600-650 μmol m^{-2} s^{-1} in Chapter 6). It is well known that the light intensity at which plants are grown affects the shape of the light-response of photosynthesis, as also shown by these two experiments, where the apparent quantum yield and the curvature degree are different and the steady-state rate of photosynthesis is attained at different light intensities. However, in both cases the dark-chilling led to similar observations. One night-chilling did not affect both the apparent quantum yield and the curvature degree, but decreased the maximal CO_2 assimilation rate. Thus, the decrease of photosynthesis at a particular light intensity after

one night of chilling is not only dependent on that light intensity value, but also on the light intensity at which plants were grown.

Results indicate that one night-chilling did not affect photosynthesis at low light intensity (200 μmol m^{-2} s^{-1}), when the synergistic effects of dark-chilling and light should have been avoided. Thus, the stress *per se* did not change the overall photosynthetic process, but turned it more susceptible to light increase, altering each species metabolism.

7.3. One night-chilling effects: electron transport chain and stomatal conductance limitations to photosynthesis at moderate and high light intensities

The electron transport processes of photosynthesis were also affected by one night of chilling in a manner that was dependent on the light intensity on the subsequent warm day (see Chapter 3), as shown by Feng and Cao (2005) for C_3 species. As for the photosynthetic rate measured at three light intensities, both the effective quantum yield of PSII electron transport (Φ_{PSII}) and the electron transport rate (*ETR*, results not shown) were generally not affected by low light intensity in any species and were just altered in *P. dilatatum* and *Z. japonica* under moderate and/or high light intensities. In accordance with the literature (Shen *et al.* 1990, Bertamini *et al.* 2006, Strauss *et al.* 2007), results show that the damages induced by dark-chilling to the Φ_{PSII} and the *ETR* in *P. dilatatum* and *Z. japonica* must have occurred at the charge separation level. This result is in accordance with the increase or tendency to increase in the non-photochemical quenching, indicating an increase in the thermal process of energy dissipation after one night-chilling. Although the decrease in *ETR* after the dark-chilling, the electron transport processes may just be limiting photosynthesis in *P. dilatatum* at high light intensities (see Chapter 3).

The whole leaf stomatal conductance to water vapour (*gs*) measured under adaxial illumination also changed after the dark-chilling in a manner that was dependent on the light intensity on the subsequent warm day (see Chapter 3 and Chapter 6). Furthermore, the *gs* changes in response to one night of chilling were generally similar to those of whole leaf photosynthesis under adaxial illumination in *P. dilatatum* and *Z. japonica*

(see Chapter 2 and Chapter 3) and both under adaxial and abaxial illumination in *P. dilatatum* (see Chapter 6), indicating that stomatal limitations to photosynthesis may exist in response to a dark-chilling stress, as also observed in the literature for C_4 plants (Pasternak and Wilson 1972, Ivory and Whiteman 1978).

An interesting result is a stomatal closure in *P. dilatatum* chilled plants at low growth light intensity when the photosynthetic rate is not affected by the imposed stress (see Chapter 3). This result shows that stomata were firstly affected by the dark-chilling stress in *P. dilatatum*, probably by inducing a water stress event at root level, as often occurs in chilling conditions (e.g. Hällgren and Öquist 1990). In a case of a water stress event, the decrease of *gs* could have resulted in the observed non-variation of leaf relative water content (*RWC*) in one night-chilled *P. dilatatum* after the rewarming of plants at low growth light intensities (see Chapter 2). The non-variation of the photosynthetic rate, *gs* and *RWC* obtained at low growth light intensity in *Cynodon dactylon* and *Z. japonica* dark-chilled plants (see Chapter 2 and Chapter 3), suggests that the possible water stress event induced by one night-chilling may not have occurred in these two species.

7.4. One night-chilling effects: carbohydrate and lipid content increased and fatty acid unsaturation degree tends to increase

Since the light intensity on the subsequent warm day influences the dark-chilling responses of plants and the aim of this Thesis is to understand the effects caused by one night of chilling *per se*, to further study the effects of this stress on metabolism the plants were grown at a low light intensity (250-300 μmol m^{-2} s^{-1}). This light intensity should avoid any interaction between dark-chilling and light effects, masking the damages on plant metabolism caused by low night temperature alone.

Results show that although photosynthesis at low light intensity was not affected by the dark-chilling in any of the three C_4 species studied (see Chapter 3), the metabolism of the plants was altered, namely the carbohydrate and fatty acid content (see Chapter 4).

After dark-chilling it was observed an accumulation of photoassimilates in the three C_4 species studied (see Chapter 4). The increase in starch content found in one

night-chilled plants (see Chapter 4) may explain the lower *SLA* and the higher *DW/FW ratio* in *P. dilatatum* and *C. dactylon* chilled plants. In *Z. japonica* control plants the level of starch was slightly higher than in the control of the other species and its increase after one night-chilling was lower, which may have resulted in the non-variation of the *SLA* and the *DW/FW ratio* after stress (see Chapter 2). The highest amount of soluble carbohydrates in *C. dactylon* under control conditions, and thus its higher leaf osmotic content, may confer this NAD-ME species a higher adaptability to low rain-fall areas, as observed by Taub (2000).

Although one night of chilling did not alter the soluble protein content of these C_4 plants in the following light period (see Chapter 4 and 6), it altered their membrane characteristics. Changes in membrane integrity could alter the activity of membrane related process, such as ATP synthesis and electron transport chain in photosynthesis and respiration. However, the increase in membrane integrity found at low growth light intensity after one night-chilling did not change the electron transport rate of photosynthesis in any of the three C_4 grasses at the same light intensity (results not shown). Although, the dark-chilling tended to increase fatty acid content and unsaturation degree in all species, two major responses could be found in terms of lipid peroxidation. While *P. dilatatum* and *C. dactylon* increased lipid peroxidation after one night-chilling, *Z. japonica* showed a decrease in lipid peroxidation suggesting that this species must have an antioxidant metabolism more activated than the others after the dark-chilling stress. Furthermore, the membrane characteristics of *Z. japonica* unchilled plants, namely a higher content of unsaturated fatty acids, may turn this species less sensitive to the chilling stress at membrane level. On the contrary, at this level the lower content of unsaturated fatty acids may turn *C. dactylon* more susceptible to the imposed stress. *Paspalum dilatatum* plants may present an intermediate chilling sensitivity of membranes among the three C_4 species.

The membrane lipid composition and peroxidation contrast with the results of gas-exchange after one night of chilling (see Chapter 2 and Chapter 3), showing that the three species may have different mechanisms to respond to a night chilling. While *C. dactylon* seems to be better prepared to respond after a short-term chilling stress, by greatly protecting the photosynthetic process, *Z. japonica* may be better prepared to respond to long-term chilling or freezing due to a higher fatty acid unsaturation and probably more flexible membranes. It is important to note that the Shangri-La variety of

C. dactylon studied persists well into the colder months accordingly to the manufacturer (Ragt Semences, Rodez, France), and the *Z. japonica* plants used were improved traditionally by breeding processes by the manufacturer (Jacklin Seed Company, Idaho, USA) to present a higher freeze tolerance.

7.5. One night-chilling effects: carboxylating enzymes activities were altered but generally did not decrease

Although photosynthesis was not affected at low light intensity after one night of chilling stress (see Chapter 3), phospho*enol*pyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activities were altered (see Chapter 4 and Chapter 6). Furthermore, as discussed in Chapter 4, the three C_4 species studied showed different PEPC and Rubisco activity responses, although generally no decreases were found.

The higher PEPC physiological activity (*Vphysiol*) in control plants in comparison to the photosynthetic rates and its increase on the subsequent warming light period after one night-chilling observed for the three C_4 species may have contributed to avoid a decrease in photosynthesis at growth low light intensity (see Chapter 3). Since, the decrease in photosynthetic rate at low temperature is though to be closely related to the decrease in Rubisco activity (e.g. Kubien *et al.* 2003), the non variation or increase in Rubisco initial activity (*Vi*) in *C. dactylon* and *Z. japonica*, respectively, may have prevented photosynthesis to decrease after dark-chilling when measured at low growth light intensity. Although Rubisco *Vi* slightly decreased in *P. dilatatum* after one night-chilling, its value was higher than the photosynthetic rates measured at low growth light intensity, either in control or in stressed plants.

The non variation of *in vitro* PEPC maximal activity (*Vmax*) and Rubisco total activity (*Vt*) in *P. dilatatum* one night-chilled plants (see Chapter 4) contradicts the decreases observed for the predicted maximal PEPC and Rubisco carboxylation rates (see Chapter 6) in the same species after one night-chilling and adaxial illumination. These results show how care must be taken when comparing data obtained *in vitro* with those from the model application.

7.6. Surface-specific regulation of photosynthesis and stomatal conductance with respect to light orientation in *P. dilatatum* leaves

The dorso-ventral regulation of photosynthesis has been characterised with respect to light orientation in dicotyledonous C_3 leaves (e.g. Syvertsen and Cunningham 1979, Terashima 1986). However, due to the symmetrical leaf structure of monocotyledonous C_4 plants and to the similar whole leaf light-response curves of *Z. mays* plants with adaxial and abaxial illumination (Moss 1964), it was expected that the photosynthetic rate was similarly regulated on each leaf surface. However, more recently and following the work of Domes (1971), Driscoll *et al.* (2006) have shown that under adaxial illumination the photosynthesis in maize is differently regulated on each leaf surface. Here we show that the different adaxial/abaxial regulation of photosynthesis and stomatal conductance is also present in *P. dilatatum* (see Chapter 5), which is a monocotyledonous C_4 NADP-ME species as maize. Furthermore, we show that the regulation of photosynthesis and stomatal conductance on the whole leaf and on each leaf surface separately in *P. dilatatum* plants is differently affected by light orientation (see Chapter 5).

Paspalum dilatatum results show that, contrary to C_3 dicotyledonous species, the absorption of light between the two leaf surfaces is similar (see Chapter 5). However, the adaxial leaf surface seems to present a lower capacity to perform photosynthesis than the abaxial leaf side. The dorso-ventral regulation of photosynthesis is not related to a different distribution of the carboxylating enzyme proteins (Rubisco and PEPC) across the *P. dilatatum* leaf, but may be related to a higher stomata sensitivity to light and to a higher ratio between the total surface area of bundle sheath cells and the total surface area of the first layer of mesophyll cells surrounding the bundle sheath on the abaxial leaf surface. In addition, it is suggested a higher PEPC activation state on this surface.

An inversion of light orientation to the leaf from the adaxial to the abaxial surface altered the photosynthetic CO_2-response curve of whole leaf and each leaf surface separately (see Chapter 5 and Chapter 6). The application of the C_4 photosynthetic model to the whole leaf photosynthetic curves performed in Chapter 6 allowed quantifying the light orientation effects on *P. dilatatum* photosynthesis. Abaxial illumination resulted in a decrease in the maximal PEPC and Rubisco carboxylation

rates (*Vpmax* and *Vcmax*, respectively) and in the maximal electron transport rate (*Jmax*) (see Chapter 6). The lower *Vpmax* and *Vcmax* obtained are not related to a different distribution of PEPC and Rubisco across the leaf (see Chapter 5). Furthermore, the lower *Jmax* value is not related with differences in the light absorption on each leaf surface (see Chapter 5). The lower steady-state photosynthetic rate under abaxial illumination in relation to adaxial illumination may thus result from the almost complete closure of stomata and null photosynthetic rate measured on the adaxial leaf surface (see Chapter 5 and Chapter 6). The adaxial stomata closure under abaxial illumination is related to the lower sensitivity of these surface guard cells to light, as observed in the literature (e.g. Turner 1970, Pemadasa *et al.* 1979, Travis and Mansfield 1981).

7.7. One night-chilling effects: adaxial/abaxial regulation of photosynthesis in *P. dilatatum* is maintained, but adaxial surface seems to be more susceptible

The decrease on the whole leaf and on each leaf surface photosynthesis after one night-chilling in *P. dilatatum* was mainly observed under adaxial illumination of the leaf (Chapter 6), as also found for *P. dilatatum* plants grown under CO_2 enrichment (Soares *et al.* 2008). Under this illumination one night chilling decreased the whole leaf *Vcmax*, *Vpmax*, *Jmax* and *Amax* estimated from application of the C_4 photosynthetic model to the whole leaf CO_2- and light-response curves.

Although the adaxial/abaxial regulation of photosynthesis is maintained after the dark-chilling, steady-state rates of photosynthesis obtained from the CO_2-response curves indicate that the adaxial leaf surface seemed to be more affected by the stress than the abaxial surface. In contrast to the literature results in response to water availability and enhanced CO_2 concentration (e.g. Turner and Singh 1984, Pearson *et al.* 1995, Wang *et al.* 1998), where stomata on each leaf surface were differently affected, dark-chilling seems to affect similarly the stomata on each leaf surface.

The results obtained from the photosynthetic curves indicate that the two leaf surfaces of *P. dilatatu*m are not functionally symmetrical, as the leaf structure suggests (Cavaco 2000), even in response to stress situations.

7.8. Sensitivity of the C_4 photosynthetic metabolism to dark-chilling

Plants with the C_4 photosynthetic pathway are considered to be cold sensitive, although some cold tolerant species are known (Sage *et al.* 1999). Among the C_4 species, the three C_4 grasses varieties/cultivars chosen in this study have been improved by the manufactures to be less cold sensitive. Our results show that although these improvements, one night of cold is sufficient to alter the plant metabolism even though no damaged had occurred to the photosynthetic rate when the light intensity on the subsequent warm day was low. The dark-chilling effects on each species studied may be related to the species and variety sensitivity to cold, as well as to the C_4 photosynthetic metabolic subtypes. Although no conclusion can be drawn on this topic, our results indicate a lower sensitivity of the photosynthetic metabolism in *C. dactylon* var. Shangri-La and a higher sensitivity in *P. dilatatum* cv. Raki and *Z. japonica* Steudel "Jacklin Sunrise Brand" plants to one night cold stress. Further investigations are needed to assess if the differences found among these three C_4 plants depend on the species or are representative of each metabolic sub-type.

7.9. Final conclusions and future perspectives

This study aimed to report the first effects of short-term dark-chilling on C_4 plants of the three metabolic sub-types. From the performed assays we may conclude that the responses of C_4 plants to a dark-chilling event depend on the number of chilling-nights, on the light intensity on the subsequent warm day and on the species studied.

At low light intensity on the subsequent warm day one night-chilling did not decrease the photosynthetic rate and the electron transport activity in any of the species, but led to metabolic changes in leaf fatty acids, carbohydrate content and carboxylating enzymes activity.

One night-chilling tend to increase leaf fatty acid unsaturation in all species. Furthermore, it increased the total leaf fatty acids content which may explain the higher membrane integrity in dark-chilled plants. In contrast with the other two species, the decrease in lipid peroxidation under dark-chilling and the higher unsaturation degree in control plants of *Z. japonica* indicate that this PEPCK species may be better prepared to respond to this stress at membrane level. Since a high level of unsaturation in the

phosphatydylglycerol class of fatty acids is usually correlated with higher levels of chilling tolerance, it would be interesting to extend the lipid composition studies in order to characterize each lipidic class. Due to the different lipid peroxidation response among grasses, it would be interesting to further study the content of the reactive oxygen species and antioxidant metabolites, as well as the activity of antioxidant enzymes and lipoxygenases.

The carbohydrate content increased in all species after one-night chilling, although a higher increase in glucose was found in *P. dilatatum* and the lower increase in *C. dactylon* plants. It would be interesting to understand if the dark-chilling has impaired (1) the carbohydrate metabolism, and thus the activity of several enzymes involved such as fructose-1,6-bisphosphatase, sucrose phosphate synthase and starch synthase, (2) the translocation and loading of photoassimilates in the phloem, and/or (3) the respiratory metabolism of sinks on the dark-period, since mitochondrial respiration after two to five hours of illumination at low growth light intensity was not affected by one night-chilling.

Carboxylating enzymes were also altered in all species after one night of chilling, either the PEPC *Vphysiol* and *Vmax* or the Rubisco *Vi* and *Vt*. Generally, an increase in PEPC *Vphysiol* was observed in all species. Since PEPC *Vmax* and Rubisco *Vt* increase only in dark-chilled *C. dactylon* plants, it would be interesting to analyse if differences in PEPC and Rubisco content have occurred in response to stress in the three C_4 species. The analysis of Rubisco activase activity and its polypeptide composition would also help to understand the different responses of Rubisco *Vi*, with a decrease in *P. dilatatum*, a non variation in *C. dactylon* and an increase *Z. japonica*. It would also be interesting to study the activity of other enzymes involved in the C_4 photosynthetic pathway, such as phosphopyruvate dikinase, NADP-ME, NAD-ME and PEPCK.

At moderate and high light intensities on the subsequent warm day after one night-chilling *C. dactylon* photosynthesis seems to be less sensitive than in the other two C_4 grasses. It would be interesting to understand if this lower sensitivity is related to an increase in the enzyme capacity and/or a higher protection of the capacity to electron transport in dark-chilled plants. Dark-chilling also showed to decrease photosynthesis and *gs* in a similar way at moderate and high light intensities, although the electron transport may just be limiting photosynthesis in *P. dilatatum* at high light intensities. The analysis of PEPC and Rubisco activity at moderate and high light intensities would

add additional information concerning the importance of non stomatal limitations to the decrease in photosynthesis in *P. dilatatum* and *Z. japonica* one night-chilled plants under these light conditions.

In addition to the referred above, it would be also interesting to have determined the effects of dark-chilling on plant metabolism just before the transference of the plants to warm temperature and to light.

Adaxial and abaxial leaf photosynthesis in *P. dilatatum* plants was differently regulated and respond differently to light orientation to the leaf. Furthermore, results indicate that the dark-chilling affected more the photosynthetic response on the adaxial than on the abaxial leaf surface. Further studies are needed to understand the metabolic differences of each leaf surface in this C_4 monocotyledonous plant and which metabolic steps on the adaxial leaf surface are more sensitive to dark-chilling. In addition, the characterization of other C_4 monocotyledonous species, belonging to the NADP-ME subtype as well as to the NAD-ME and PEPCK subtypes, and the comparison of the photosynthetic adaxial and abaxial responses of C_4 species with C_3 and C_3-C_4 plants needed to be performed. These results may be of major evolutionary interest.

From this Thesis results it may be suggest that, among the C_4 species studied, *C. dactylon* is the more suitable to use in a scenario of increasing daily temperatures with short-term periods of low night temperatures. However, studies with a higher number of low night temperatures will be needed in order to have a better understanding of the effects of this stress on photosynthesis. Furthermore, the results obtained suggest that the changes induced by one night-chilling in the photosynthesis in *P. dilatatum* and *Z. japonica* are similar to those induced in chilling-sensitive C_3 plants, at least those concerning stomatal conductance and chloroplast electron transport. In addition, the results presented in this Thesis regarding adaxial and abaxial regulation of photosynthesis and stomatal conductance in *P. dilatatum* and the effects of dark-chilling on that regulation, together with those of a work already published by Soares *et al.* (2008) with plants grown under CO_2 enrichment, opens a new area of research in C_4 plants.

7.10. References

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